Vol. 49, No. 4 December 1961

THE ANNALS OF APPLIED BIOLOGY

EDITED FOR THE ASSOCIATION OF APPLIED BIOLOGISTS

BY

R. W. MARSH

AND

I. THOMAS

WITH THE ASSISTANCE OF

F. C. BAWDEN J. T. MARTIN
M. J. R. HEALY E. A. PARKIN
D. J. WATSON

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price Thirty Shillings net

The Association of Applied Biologists

CONTENTS OF Vol. 49, No. 4

Ann. appl. Biol. (1961), **49**, 571–577
Printed in Great Britain

The poison baiting of corn-ricks with particular reference to the control of house-mice

By F. P. ROWE, E. J. TAYLOR and A. H. J. CHUDLEY
Infestation Control Laboratory, Ministry of Agriculture, Fisheries and Food, Tolworth, Surrey

(*Received* 13 *March* 1961)

SUMMARY

Experimental warfarin treatments designed to reduce house-mouse (*Mus musculus*) and common rat (*Rattus norvegicus*) populations in corn-ricks are described. Baiting rick bases at permanent poison points or in rat holes was found not to be completely successful in controlling either rats or mice. Bait placed 1 ft. inside ricks in containers or wrapped in paper was effective in reducing the level of mouse infestation. In ricks treated after erection, baits wrapped in paper were more easily applied.

Samples of threshed grain taken from each sack showed that ricks baited internally contained fewer mouse droppings but more poison bait residues than ricks baited at ground level only.

INTRODUCTION

Owing to the increasing use of combine harvesters, far fewer corn-ricks are being built in England and Wales than there were, say, 10 years ago. It seems likely, however, that some rick building will continue into the foreseeable future and in Scotland over half of the cereal crop is still stacked (Kent, 1959).

Corn-ricks unfortunately are extremely vulnerable to rodent infestation, and the control of rats (*Rattus norvegicus*) and particularly of house-mice (*Mus musculus*) living in them is a long-standing problem. In the past various methods have been tried to reduce damage. These include staddles (Kent, 1953), repellents (Chitty & Southern, 1954), fumigation (Chitty & Southern, 1954; Thompson, 1959), and barriers, but none has proved entirely satisfactory.

Most of the work carried out by the Bureau of Animal Population, Oxford University, during the 1939–45 war, on the control of rats and mice in corn-ricks was concerned with poison baiting. Chitty & Southern (1954), during field trials on poisoning rats in ricks with various acute poisons, noticed that a few house-mice were also killed. In later experiments against mice, hundreds of small piles of poison bait were pushed into mouse-holes found in the sides of infested ricks. Of the poisons used, zinc phosphide gave the best results, with kills of the order of 50–90%, but owing to the danger of bait residues, this method could not be recommended.

Renewed interest in the control of rats and mice in corn-ricks by poisoning followed the advent of warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin], for by careful use of this 'chronic' rodenticide the importance of any toxic residues left in the grain after

threshing can be much reduced. Even so, during the 7 or 8 years in which warfarin has been employed, baiting has been largely confined to the entrances of rat-holes in the ricks: and any reduction of rat populations achieved may have aggravated the mouse problem by making it easier for mice to increase. Thus, there is a need for improved poison baiting techniques against mice in ricks.

Recent experimental work has been concentrated on preventing the build-up of mouse populations in ricks by using permanent warfarin baiting points (J. H. Cuthbert, Department of Agriculture and Fisheries for Scotland, private communication; Rowe & Chudley, unpublished) rather than poisoning after infestation has occurred—the method adopted with acute poisons. In trials in 1959–60 two permanent baiting methods were used and there follows an account of the results obtained.

METHODS

The corn-ricks chosen for the experiments were built singly or in groups in fields on a farm at Odiham, Hampshire. In all, thirty-seven ricks were used in four experimental trials (A, B, C and D). The bait used was pinhead oatmeal plus 5% sugar and 5% technical white oil, and it contained 0·025% warfarin.

Trial A. Eleven ricks were permanently baited primarily against rats. Around each rick twenty equally spaced ½ lb. bait points were laid externally. They were inspected regularly at monthly intervals, when the numbers of rat and mouse pellets were counted and removed and the bait weighed. Bait which had become mouldy or dirty was replaced. Open rat or mouse-holes discovered around the base of the rick were noted and a record kept of any dead animals found.

Trial B. As in trial A, a further twelve ricks were treated externally round the base, against rats. In addition, however, in an attempt to control house-mice, ½ lb. baits (between 30 and 40) were placed inside the ricks a few days after they were built. Cylindrical bait containers (9 in. long and 3 in. in diameter) made of metal or cardboard were used. Each had one end closed by a lid and the other left open. The containers were pushed into the sides of the rick, parallel with the lie of the sheaves, so that the open ends were facing outwards and were 3–6 in. inside it. They were sited in two staggered rows, one just below the eaves and the other halfway between the base and eaves. The distance between containers in the same row was 5 ft. Every 2 months the condition of the bait was checked and fresh bait replaced, if necessary. Rodent droppings in the bait and container were counted and removed.

Trial C. In this trial seven ricks were treated for rats by the local authority's rodent operator. Each treatment consisted of a monthly inspection of the ricks and a few yards of the hedgerow (if any) near the rick site, followed by the baiting of any rat holes discovered with 0·005% warfarin in medium oatmeal.

Trial D. Six ricks, besides being hole-baited against rats by the local authority, were also treated against house-mice with baits prepared by wrapping 2–3 oz. of bait in a piece of rough writing paper, as practised by Cuthbert in Scotland. Ricks were first baited with paper baits 4–5 weeks after being built. The baiting technique was similar to that with the metal or cardboard containers, each rick having two rows of baits laid at 5 ft. intervals. The paper baits however were pushed further into the rick (to about 12 in.) and were not re-examined until threshing took place.

Census and sampling techniques

Immediately before each rick was due to be threshed a rodent-proof hessian barrier was erected around it. House-mice were caught by hand and then chloroformed; rats were killed by violence. After threshing, the straw base of each rick was raked over to find hidden animals. Occasionally mice entered the threshing machine and came out on to the riddle; these and the few live animals which were seen to escape were included in the total numbers found in the rick.

The level of rodent contamination and the presence of bait residues in the threshed grain were determined by taking a sample of the first-grade grain as each sack was filled. The sample (about 1 lb.) was caught in a tin placed between the chute and the sack, weighed, tipped on to a white tray and examined for rat and mouse droppings and for bait. No chemical analysis was attempted and only the larger bait particles may have been detected.

RESULTS

Effectiveness of the four control methods

The numbers of live and dead rats and mice found in each rick at threshing are given in Table 1. This shows that no rick was heavily infested (maximum population, 221 mice), but none was completely free of mice. It also shows the considerable variation in the number of animals taken from ricks treated in the same manner. As there is no evidence of significant differences dependent on the type of grain in the ricks, Table 1 includes the totals for all the ricks in each respective treatment.

The comparative effectiveness of the control methods is more obvious when the average number of live mice per rick at threshing is related to the type of treatment (Table 2). The average number of live animals found in ricks baited internally (trial B) is significantly less than in those baited externally only (trial A: $P = 0{\cdot}02$–$0{\cdot}01$): there is also a significant difference when trials C and D are compared ($P = 0{\cdot}001$).

Monthly counts of droppings in the bait containers

During wet weather it was found impossible to keep bait dry in the external containers at the base of the ricks and this prevented any accurate estimation of consumption. The numbers of mouse droppings found in these containers each month, however, give a rough guide to the monthly variation of mouse activity, this being greatest between September and December, least between January and April, and then increasing slightly in May and June.

The bi-monthly counts of droppings for individual ricks showed that there was some variation in mouse activity between the two levels at which the internal bait containers were situated. The total number of droppings found in the containers inserted into the sides of twelve ricks (trial B) is given in Table 3. The figures probably include some droppings of harvest-mice. These animals were found along with house-mice in eight of the ricks and it was not easy to differentiate between the excreta of the two species. More droppings were found in the upper than in the lower rows of containers. The difference ($88{\cdot}7 \pm 77{\cdot}5$) however is not significant.

The number of droppings found in the containers (Table 4) reached a maximum

between October and December, fewer being found in later months. The number of mice live-trapped around four other ricks formed a similar pattern, more animals being captured in October than any other month. It would seem that the baiting technique was effective in depressing rather than eradicating the mouse population.

Table 1. *Total rick populations of mice and rats at threshing*

Cereal*	Standing time (weeks)	House-mice		Rats	
		Alive	Dead	Alive	Dead
	Trial A. Ricks baited externally at the base only				
O	18	22	0	0	0
B	28	8	5	0	0
W	28	7	1	0	1
W	46	3	3	21	0
W	44	1	0	0	2
B	30	4	7	0	0
W	38	221	18	0	0
W	40	128	31	2	1
W	43	220	31	2	3
W	40	84	2	0	2
W	39	54	39	4	1
		752	137	29	10
Trial B. Ricks baited externally at the base and internally with metal or cardboard containers					
O	25	33	28	0	5
O	9	3	0	0	0
O	35	5	8	0	2
B	28	1	5	0	0
W	45	6	14	1	2
W	44	1	1	2	6
B	30	3	2	0	2
W	37	14	9	6	0
W	38	12	8	0	0
W	40	29	21	1	0
W	40	17	12	0	1
W	39	2	4	1	0
		126	112	11	18
	Trial C. Ricks hole-baited for rats only				
B	32	22	0	0	0
B	27	45	4	1	0
B	37	68	0	0	0
W	35	21	5	0	2
W	39	92	15	5	0
W	28	47	0	4	0
W	41	53	1	0	0
		348	25	10	2
	Trial D. Ricks hole-baited for rats and internally with paper baits				
B	31	7	4	0	0
B	27	32	6	0	0
B	39	1	5	0	3
W	40	2	12	1	0
W	24	1	13	0	0
W	37	7	12	0	0
		50	52	1	3

*B = barley; W = wheat; O = oats.

Contamination of threshed grain with rodent droppings

The relationship between the different control measures practised and the degree of contamination of the threshed grain with rat and mouse droppings is shown in Table 5.

The average number of mouse droppings per 1 lb. of grain in ricks baited internally (trial B) is significantly less than in those baited externally only (trial A) ($P < 0.001$) and the difference when Trials C and D are compared is also significant ($P = 0.001$–0.01).

Table 2. *The average number of mice per rick related to type of poison treatment*

Baiting method	Number of ricks	Average time of standing (weeks)	Average number of live mice/rick
(A) Base	11	35·9	68·4
(B) Base + internal (cylinders)	12	34·2	10·5
(C) Hole	7	33·7	49·7
(D) Hole + internal (paper)	6	33·0	8·3

Table 3. *Total numbers of droppings found in internal bait containers*

Rick ...	1	3	5	6	10	11	14	15	17	18	21	23	Mean
Top row	280	174	274	871	295	193	738	406	232	305	501	539	400·7
Bottom row	500	27	260	263	365	165	512	416	231	154	408	443	312·0

Table 4. *The average number of droppings per rick in internal bait containers at bi-monthly intervals*

Month inspected ...	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June
Number of ricks	6	6	6	5	6	5	6	3	5	2
Average number of droppings per rick	128	165	283	283	186	209	129	31	83	13

Table 5. *The average number of rat and mouse droppings per 1 lb. of grain related to poison-baiting method*

Baiting method	Number of ricks	Average standing time (weeks)	Total weight of grain samples (lb.)	Average number of droppings per 1 lb. grain	
				Mouse	Rat
(A) Base	11	35·9	861	4·90	1·49
(B) Base + internal (cylinders)	12	34·2	1,138	0·63	0·47
(C) Hole	6	33·7	563	7·76	0·41
(D) Hole + internal (paper)	6	33·0	525	0·79	0·04

In considering the level of contamination it is interesting to group ricks according to population size and measure the corresponding degree of contamination of the threshed grain (Table 6).

Contamination of threshed grain with warfarin bait

No warfarin bait was found in the samples taken from sixteen of the twenty-three ricks poisoned at the base only, or from the only three ricks baited above ground level by the local authority. Bait was discovered in samples taken from five out of twelve ricks having internal bait containers and four out of six treated with paper baits. However, of a total of 1646 samples of grain examined only 2·9% had visible bait particles. Samples from ricks treated with paper baits were more contaminated (6·6%) than those taken from ricks with containers (1·6%).

Table 6. *The relationship between the numbers of mice in ricks and the level of grain contamination*

Number of live mice at threshing	Number of ricks	Average number of mice per rick	Average number of mouse droppings per 1 lb. of grain
0–25	23	7·4	0·66
26–50	5	37·2	1·96
51+	7	121·7	11·15

DISCUSSION

A clear-cut comparison of the efficiency of the four methods of control is difficult in the absence of figures from untreated ricks and in view of other factors such as the numbers of free-ranging rodents in the vicinity when the corn is cut, the type of grain harvested, rick size, incidence of predators and time of threshing—all of which may influence population size and which may vary from site to site.

The numbers of dead mice found in ricks treated permanently with bait laid either around the base only (trial A) or in rat holes (trial C) suggest, however, that these methods have some effect in limiting house-mouse populations. For although natural mortality may have accounted for some dead animals it is significant that most were found in the lower half of the rick and in the straw base near the bait points. Moreover, autopsy of some of the dead mice (not included in Table 1) that were picked up during bait inspection in trials A and B showed that death was almost certainly due to warfarin poisoning.

Although the number of ricks treated was small, the results of trials B and D (Table 2) give an indication of the additional degree of mouse control that can be expected if ricks are baited internally. The two internal baiting methods that were tried appeared to be equally effective. Irrespective of type of control treatment, the grain was more contaminated with mouse droppings above than below eaves height and it is probable that additional baits above eaves height would have resulted in still better control.

There is evidence to suggest that for the best results, baiting must be practised during rick building or, at any rate, very soon afterwards. The relative ease with which bait can be applied is a practical consideration which is linked, in our experience, with the time at which baiting is attempted. While ricks are being built, both baits in paper

and in containers are equally easy to apply. In ricks treated after erection, however, paper baits were easier to use. Whereas in the trials described it was a simple operation to thrust these into the sides of completed ricks, the same operation employing containers proved difficult and time-consuming, even in ricks erected only one week previously. For this reason, and because of the extra cost of obtaining and transporting containers, the paper-bait technique would seem to be preferable.

Of the fifty-seven dead mice picked up during bait inspections, 65% were found in September and October, suggesting that mice were most numerous around the base of the ricks during these months. This is supported by figures of the monthly counts of droppings in the bait containers and evidence from live trapping: also by the statement of Southern & Laurie (1946) that in the autumn fair numbers of house-mice are found round new ricks and out in the hedgerows, but that later in the year and early spring, considerably smaller numbers are found, mainly in the hedgerows.

It was apparent during the monthly bait checks (trials A and B) that some mice were living beneath the straw base of the rick, earth from their runways sometimes covering the bait trays. Removal of the straw base after threshing revealed that these runways often penetrated 4–5 ft. horizontally underneath a rick. Little is known about the range of mice living in the ricks themselves, but it is believed that there is less movement up and down than horizontally (Southern & Laurie, 1946). The better control obtained with internally placed baits is therefore probably because these extra baits help to kill mice which had either been carried into the rick or entered the upper half without encountering the poison bait laid round the base.

It was not unexpected that some warfarin bait would find its way into the threshed grain. Occasionally, during the periodic removal and examination of the baits in containers, slight spillage occurred, while approximately one half of the paper baits were not recovered at threshing, despite close attention and the co-operation of farm hands. Uneaten baits were sometimes lifted together with sheaves into the threshing machine and broken up in the drum. It is difficult to assess the risks involved and further work on this aspect is being undertaken.

We are indebted to Mr C. Saunders of Roke Farm, Odiham, Hampshire, for allowing us to carry out this work on his farm, and for his generous assistance. It is a pleasure to acknowledge the friendly help of the threshing team, and of our colleagues R. Redfern and W. Rayner; and to thank Hartley Wintney U.D.C. for their close co-operation.

REFERENCES

Chitty, D. & Southern, H. N. (1954). *Control of rats and mice*, 3 vols. Oxford: Clarendon Press.

Kent, N. L. (1953). Wastage on the farm: staddles for corn stacks. *Fmg News, Glasg.* **105**, 10.

Kent, N. L. (1959). Contamination of stacked grain by rats and mice. *Scot. Agric.* **39**, 98.

Southern, H. N. & Laurie, E. M. O. (1946). The house-mouse (*Mus musculus*) in corn-ricks. *J. Anim. Ecol.* **15**, 134.

Thompson, R. H. (1959). Fumigation against mice. *Pest Technol.* **2**, 7.

Ann. appl. Biol. (1961), **49**, 578–587
Printed in Great Britain

Comparison between malathion spraying and lindane–whitewash mixture for controlling *Ephestia elutella* (Hübn.) in warehouses

By MARGARET T. ARMSTRONG
Ministry of Agriculture, Fisheries and Food, Infestation Control Laboratory, Liverpool

(*Received* 16 *March* 1961)

SUMMARY

Provisional estimates of the total numbers of adult *Ephestia elutella* emerging from warehouse rooms of about 4000 cu.ft. were between 228,300 and 509,300 in 'moderately infested' rooms and 1,670,000 in a 'heavily infested' room.

Moths caught on commercial fly-papers are a useful index to the emergence date of adult *E. elutella*.

A trial in a Liverpool warehouse in 1958 showed that lindane in a commercial whitewash mixture, applied to walls and ceilings to produce an initial deposit of 40 mg. γ-BHC/sq.ft., will remain effective against *E. elutella* between May and December. To obtain this deposit it was necessary to apply over twice this dosage. Very few active moths were seen and a negligible larval migration took place from trap bags which had been the only foodstuffs stored there.

Observations over the same period in a warehouse which had been treated commercially at a heavy dosage, in December 1957, showed similar results.

Cage tests in the laboratory and in the field indicated that malathion water-dispersible powder, applied at an initial deposit of 162 mg. malathion/sq.ft., would theoretically be as satisfactory as lindane–whitewash mixture. In warehouses, however, the former was not so effective as the latter, probably because of the marked fumigant effect of gamma-BHC.

There were indications that lindane–whitewash mixtures alone might control infestations of endemic *E. elutella* in fully loaded rooms.

INTRODUCTION

Armstrong & Hill (1959) showed that lindane–whitewash mixtures applied to walls were effective as a supplement to *inefficient* spraying of commodities with pyrethrins for the control of the warehouse moth *Ephestia elutella* (Hübn.).

The present paper describes an experiment on the effects of wall treatments with lindane in a whitewash mixture, and with malathion as a spray. The latter was included in the tests because preliminary laboratory work and other information (Parkin, 1958) had indicated that malathion had long-lasting effects against moths.

METHODS

The experiment was carried out in two typical adjoining Liverpool warehouses, between May and December 1958. The stores had been infested with *E. elutella* for some years and had been empty since December 1957.

In warehouse A, room 1 (ground floor), the walls and ceilings were sprayed at the beginning of the experiment with lindane–whitewash paste, using a lime-washing machine, at a theoretical dosage of 103 mg. γ-BHC/sq.ft. Room 3 (second floor) was sprayed, using a knapsack sprayer, with malathion water-dispersible powder, at a theoretical dosage of 162 mg. malathion/sq.ft. Room 2 (first floor) and room 5 (top floor) were not sprayed. Room 2 acted as a control and room 5 was used for occasional observations.

The walls of warehouse B (ground, first, second and third (top) floor) had been sprayed by the owners with lindane in whitewash, in December 1957. The dosage used is not known, but later chemical tests suggested that it must have been about 250 mg. γ-BHC/sq.ft.

Before moth emergence fifteen coarsely woven hessian sacks, each containing 50 lb. fumigated groundnut sweepings, were placed in rooms 1, 2 and 3 of warehouse A and in rooms 2, 3 and 4 of warehouse B. In each room the bags were set out in three rows of five across the middle third of the room.

In the warehouses the walls and bags were examined weekly and observations or counts were made. On two occasions dead larvae and dead adults on seventy-eight 1 ft. square areas on the floor were counted. At the end of August the bags were removed to the laboratory where each bag was isolated by sticky banding and examined for migrating larvae.

Adults trapped on ten or twelve commercial fly papers hung in room 5, warehouse A and rooms 2–4, warehouse B, were counted weekly.

Cage tests with *E. elutella* adults (Armstrong & Hill, 1959) were made in rooms 1–3 in warehouse A at intervals. Strips of filter-paper, which had been placed in position at the time of the original spraying, were removed on each occasion for chemical estimation of the spray deposit.

POPULATION STUDIES

(*a*) *Overwintering larvae*

Although it had been anticipated that the spraying would not have any effect until the moths emerged, many dead larvae were soon seen on the floors, mainly at the base of the walls (Table 1).

Table 1. *Larvae dead on floors 6 weeks after spraying*

Warehouse A	On 78 areas each 1 sq.ft.	Mean per sq.ft.		Calculated number on total floor area
		Base of walls	Remainder	
Room 1 (lindane)	2,160	90·4	4·5	38,313
Room 2 (control)	120	3·9	0·5	2,598
Room 3 (malathion)	762	28·4	1·94	13,663

Counts were made at 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 105 ft. along the complete long axis and at 0, 4, 8, 12, 14 and 16 ft. from the centre of the room to the walls (2·4% of total area) in rooms 1–3 of warehouse A. The results indicated either that the insecticides stimulated diapausing larvae into movement or that a certain proportion of the population was active; wandering larvae were seen from time to time during routine inspections in winter and spring.

The examination of three areas of walls (Table 2) gave a further indication that larvae had been killed by the spray residue.

Table 2. *Larvae and pupae on walls*

(Counts made on three areas of wall (4·1 % of total area).)

	Spun-up larvae		Walking	Pupae	
Warehouse A	Alive	Dead	larvae	Alive	Empty cases
Room 1 (lindane)	10	29	2	13	2
Room 2 (control)	14	10	3	162	5
Room 3 (malathion)	2	19	2	12	1

(*b*) *Adults trapped on fly papers*

Moths were trapped first in the two top rooms, on 27 May in warehouse B, and on 3 June in warehouse A, but were not seen flying or on the structure until 2 weeks later. In the lower rooms a very few moths appeared on fly papers on 10 June in room 3, warehouse B. By 17 June they were seen flying and on the structure in all rooms. The difference in times of emergence between upper and lower floors had been noted during routine inspections and was probably due to higher temperatures in the top rooms, many of which had unlined slate roofs, with numerous glass roof lights.

The total numbers caught on fly papers are shown in Table 3.

Table 3. *Moths caught on fly papers*

Warehouse A		Warehouse B (lindane)	
Room 5 (top) (unsprayed)	11,726 (May–October)	Room 4 (top)	1,385 (June–October)
		Room 3	59 (June–July)
		Room 2	42 (June–July)

A much greater number was found in the top room (room 5) of warehouse A than elsewhere. This room had held no foodstuffs and had not been treated with insecticide. It is believed that the fly papers caught most of the adults in the room. Visual observations confirmed that the peak emergence was between 1 and 14 July and only small numbers were ever seen on the floors.

All rooms in warehouse B had been treated with lindane. There was no very marked peak emergence and the moths caught in the top room were only a fraction of the probable total number present, which was about 1,500,000 (Table 6).

(*c*) *Adults found on walls, window ledges and groundnuts*

The numbers of live adults found each week on walls (including windows and doors, representing 16·5% of the total wall area) and on the flat surfaces of bags of groundnuts in rooms 1–3 of warehouse A are shown in Figs. 1 and 2.

Maximum numbers on walls were found on 14 July, but the peak number on the control bags did not occur until a week later. The number of living adults counted in the treated rooms of warehouse A was much less than in the control. There was a well-marked peak on 14 July in the 'malathion room' but the number in the 'lindane room' was always small. The relatively small number of moths observed on the trap bags in the treated rooms showed that the wall treatments had the effect of preventing all except a small proportion from reaching the bags (Table 4). Lindane had a more pronounced effect than malathion.

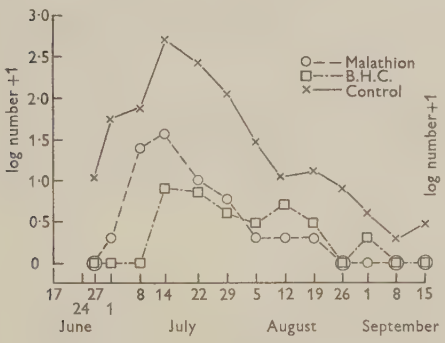


Fig. 1

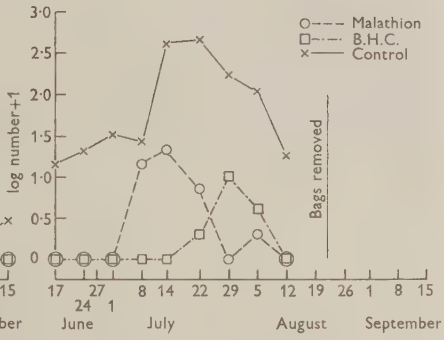


Fig. 2

Fig. 1. Numbers of live moths on walls.

Fig. 2. Numbers of live moths on trap bags.

Table 4. *Moths observed on groundnut trap bags*

Warehouse A	*Live*	*Dead*
Room 2 (control)	1283	2035
Room 3 (malathion)	40	148
Room 1 (lindane)	13	132
Warehouse B (lindane in 1957)		
Room 4 (top)	17	172
Room 3	5	142
Room 2	13	335

Living moths were first seen on the bags in the control room. It was not until 2 or 3 weeks later that dead moths were seen on the bags in the malathion- and lindane-treated rooms. In each case living moths were first seen on the bags a week later.

Further evidence of reduced activity caused by the insecticides was obtained from counts on window ledges (50% of the total ledge area) (Table 5).

The greatest increase in numbers was between 14 and 22 July, i.e. after the peak period of emergence.

Table 5. *Moths on window ledges*

Warehouse A	Live	Dead	Warehouse B	Live	Dead
Room 2 (control)	73	69	Room 3 (lindane)	0	542
Room 3 (malathion)	3	40	Room 2 (lindane)	0	663
Room 1 (lindane)	0	286			

(*d*) *Total adult population*

The level of infestation in the rooms, in May 1958, had been estimated by inspection (Freeman, 1948). Rooms 1–3 in warehouse A and 2–3 of warehouse B were judged to contain 'moderate numbers of spun-up larvae' and to be 'moderately infested'. Room 4 of warehouse B had 'fair numbers of larvae' and was 'heavily infested'.

An indication that rooms 1–3 in warehouse A were similarly infested was given by counts of spun-up larvae around the tops of pillars (13·5% of area). There were fifty-two larvae in room 1 and forty-six in room 2 and in room 3.

When it was clear that most of the moths had emerged, counts were made of those on the floors (Table 6). The same seventy-eight 1-sq.ft. areas (Table 1) were used as for the counts of dead larvae. A count of dead moths was also made in the badly infested top room of warehouse B.

Table 6. *Dead moths on floors*

Warehouse A	Total no. on seventy-eight areas each 1 sq.ft.	Mean per sq.ft.	Calculated for total floor area
Room 2 (control)	6,172	79	265,863
Room 3 (malathion)	5,295	68	228,000
Room 1 (lindane)	11,825	152	509,300
Warehouse B			
Room 4 (lindane) (top)	38,758	497	1,670,000

Richards & Waloff (1946), in their comprehensive study in a London bulk grain warehouse of similar dimensions, report estimated populations of about 300,000. Warehouse A seems to have had similar numbers, but the top room of warehouse B had a very much greater population.

(*e*) *Larval production*

Migration of larvae from the trap bags (which had been removed to the laboratory on 28 August) began on 1 September and reached a peak during the week beginning 20 October. No larvae were seen in the warehouse even though inspection continued until December. At the final examination of the bags in January 1959, very few larvae were found inside the nuts although a number had spun up on the fabric of the bags (Table 7).

The small number of larvae which had developed in the trap bags in the lindane-treated rooms is a clear indication of the effectiveness of this method of control.

Table 7. *Total adults. Migrating, and spun-up larvae from trap bags*

Warehouse A	Migrating larvae: Adults (Table 4)	Migrating larvae: From fifteen bags	Migrating larvae: Mean per bag	Spun-up larvae: Sieved from five bags	Spun-up larvae: Spun-up on fifteen bags
Room 2 (control)	3,318	33,953	2,264	38	3,279
Room 3 (malathion)	188	4,275	285	0	553
Room 1 (lindane)	145	34	2·3	3	48
Warehouse B					
Room 4 (lindane)	189	215	14	—	12
Room 3 (lindane)	147	302	20	—	11

CHEMICAL ESTIMATION AND FATE OF INSECTICIDE DEPOSITS

The mean deposits of the insecticides in warehouse A are given in Table 8. There were four lindane and five malathion wall sites respectively, and two ceiling sites, and there were one to three replicates at each site. The methods of analysis were: γ-BHC by Volhard's method in Howard (1947) and malathion as in Norris, Vail & Averell (1954). The analyses were done by E. G. Hill, B. S. J. Border and W. Simpson of the Ministry's Liverpool Chemical Laboratory.

Table 8. *Mean deposits of insecticide in mg./sq.ft. (with S.E.). Walls sprayed* 6 *May*

	γ-BHC					
Test dates ...	6 May	3 June	15 July	2 Sept.	14 Oct.	3 Dec.
Test no.	—	A	B	C	D	E
Days since spraying	0	28	70	119	161	211
Mean deposit	40·8 ± 11·73	27·5 ± 14·19	7·7 ± 9·61	2·75 ± 3·18	1·49 ± 1·06	1·9 ± 1·08

All determined from deposits on filter-papers secured to the walls except E, from wall scrapings.

	Malathion					
Test dates ...	7 May	10 June	22 July	9 Sept.	21 Oct.	16 Dec.
Test no.	—	A	B	C	D	E
Days since spraying	1	35	77	126	168	224
Mean deposit	138·5 ± 73·5	72·6 ± 26·0	69·03 ± 31·6	47·8 ± 16·3	82·2 ± 30·6	86·6 ± 42·8

Since the application of γ-BHC was made at 103 mg./sq.ft. (p. 579) the amount lost during spraying was 62%. This confirmed previous observations showing losses between 50% and 70% (Armstrong & Hill, 1959). Only 14% of malathion, applied at 162 mg./sq.ft., was lost. The difference in loss during spraying was probably mainly due to the different spraying techniques used.

The lindane mixture (commercial whiting paste containing lindane powder, whiting, sticker and water) was applied with a lime-wash sprayer. Sedimentation occurred in spite of the built-in paddle stirrer and hand stirring and some of the mixture remained behind in the machine. It was known that this type of lime-washing

nozzle clogs with thick mixtures. The volume of the spray mix was therefore adjusted so that two applications to the walls would give the calculated dosage. The 'run-off', due to the large output of the spray nozzle, was therefore doubled, and accumulated on the floor and fillets at the base of the walls. Because of the large output of the spray nozzle the walls were sprayed from a greater distance than with the knapsack sprayer used for malathion. The losses as 'fall-out' would therefore be correspondingly greater (Yeomans & Van Leeuwen, 1954).

The malathion water-dispersible powder was applied with a pressure-retaining knapsack sprayer. Although some sedimentation may have occurred no insecticide remained behind in the sprayer. There was less 'run-off' from the finer spray nozzle. Nevertheless, deposits measured near the base of the walls were $1\frac{1}{2}$–3 times greater than those at the middle and top of the walls. There was less chance of loss as 'fall-out' from the fine spray nozzle, which was held closer to the walls.

CAGE TESTS WITH INSECTS

The technique was that described by Armstrong & Hill (1959). There were five replicates in the insecticidally treated rooms and three in the control room. Mortality figures were analysed statistically. Only those which were significantly greater than the controls (P better than 0·05 by Student's t test) have been used in Fig. 3 except for malathion, Test E, where an intermediate result is included and indicated by a broken line. Test D, malathion, has been omitted, since only two of the results were significant.

Results with lindane were very similar in all tests, 70% mortality being achieved 4 days after exposure. Armstrong & Hill (1959) reported that this mortality was usually achieved 3 days after exposure, from similar deposits, but different formulations. Attention was also then drawn to the problem of different physical properties of these lindane–whitewash mixtures.

With malathion the results were more variable, for 68% mortality occurred 3 days after tests A, B, C (June, July, September), but only 56% mortality was reached 6 days after exposure in test E (December), despite the fact that there was nearly as much malathion present. In practice this would be of little consequence since the main moth emergence occurs in July and it is control in the early period of emergence that is required.

The results obtained with malathion and lindane were compared statistically, using Student's t test. In some of the earlier tests malathion produced higher mortality than lindane, but at the later tests the reverse was true. The significant results (P better than 0·05) are indicated in Fig. 3.

It is not clear why this apparent changeover took place. That it was not due to differential effects of temperature is shown by the fact that although the temperature at the time of test E was 42° F. it was 57° F. at test D, and this was only 1° F. lower than it had been during test A.

A more significant finding is, however, that the cage tests indicated that malathion should have been at least as effective as lindane in killing adults, but the warehouse observations showed clearly that lindane gave much better results in practice, perhaps

because malathion is principally a contact insecticide, whereas γ-BHC also acts as a fumigant. Armstrong & Hill (1959) showed that moths subjected only to the fumigant effect of γ-BHC died almost as quickly as when they could also have direct contact with the insecticide.

When moths were tested for fumigant effect over a malathion-treated surface, at 77° F. and 70% R.H. the differences in mortality from when the moths were placed in direct contact with the insecticide were about 20%, 40% and 60%, 1, 2 and 3 days respectively after exposure (mean deposit 108·2 mg./sq.ft.) (Table 9).

The greater efficacy of malathion as indicated by the test-cage experiments suggests

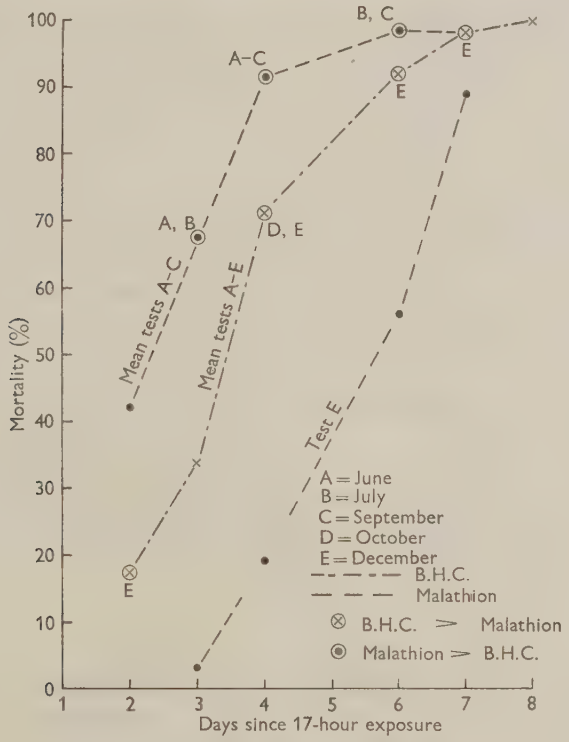


Fig. 3. Adult mortality: cage tests.

Table 9. *Malathion. Comparison between mortality* (%) *of moths exposed to direct contact and fumigant effects* (*control values subtracted*)

	Days since exposure		
	1	2	3
Direct contact	24·8	58·2	78·5
Fumigant	1·5	19·5	19·2
Difference	23·3	38·7	59·3

that the caged moths had more opportunity to pick up a lethal dose than did the warehouse population.

Studies are now proceeding on the effects of γ-BHC on the pupae and on newly emerged adults. Preliminary results are mentioned in the discussion.

DISCUSSION

In field trials of this nature it is important, if possible, to work with similarly infested sites for different treatments. It had been thought that the three main experimental rooms in warehouse A were all moderately infested. Numerical assessment of larvae spun-up in the walls proved to be impossible because no sound criterion could be devised. Methods of trapping, e.g. suspending muslin bags (Richards & Waloff, 1946) would have been difficult in empty rooms and might have prejudiced the results. It was felt that the cumulative floor count of dead adults would give a reasonable indication of the population, because all three rooms were similar in size, shape and structure (except that room 1—lindane—had a concrete instead of a wood floor).

The floor counts indicated that rooms 2 (control) and 3 (malathion) were similarly infested and that room 1 (lindane) contained nearly twice as many moths. A number of larvae had died naturally or been killed by the insecticide, but even in the lindane room, where mortality was highest, it could have reduced the eventual population by only about 6%, and this can be ignored. Furthermore, although many more adults remained on the trap bags in the control room than elsewhere, they represented under 1% of the probable total population.

Table 10. *Ratio of moths recorded and larvae produced from trap bags*

Warehouse A	Moths	Larvae	Ratio (No. of larvae per moth)
Room 2 (control)	3,318	37,232	11
Room 3 (malathion)	188	4,838	26
Room 1 (lindane)	145	82	0·6
Warehouse B			
Room 4 (lindane)	189	227	1·2
Room 3 (lindane)	147	313	2·1

It is unlikely that the insecticidal treatment caused the difference in numbers between the lindane-treated room and the others in warehouse A by affecting the behaviour of the moths. The explanation must be sought in the previous history of the warehouse. The large population makes the success of the treatment all the more striking.

Although small numbers of larvae developed on the trap bags in the malathion- and lindane-treated rooms, quite a number of moths were recorded on them and even larger numbers in the rooms as a whole (Tables 4 and 6). If we consider the ratio of moths recorded and larvae produced (Table 10) we observe that this is highest for the malathion-treated room and lowest for the lindane-treated room, the control room being intermediate.

If we assume that the sex ratio was the same in the three rooms, the ratios quoted

above could indicate that the fertility of the moths in the lindane-treated room had been adversely affected or that there was some effect on the young larvae. The high ratio for the malathion-treated room could mean that the moths which reached the bags were not affected by the insecticide or had possibly avoided contact with it. On the other hand, the malathion could have stimulated the fertility of those moths which had escaped unharmed from the walls or room.

The results of the main experiment indicate clearly that the spraying of walls with lindane mixtures alone can effect a considerable reduction in the population of *E. elutella* and can prevent serious infestation of goods stored in treated rooms.

Numerous colleagues assisted in the warehouse or laboratory work of whom the author wishes to thank particularly M. M. Senior, E. G. Hill, K. G. Smith, Mrs L. A. Walton, B. S. J. Border, W. Simpson and Mrs S. M. Collins.

The Warehouse Company concerned is also thanked for providing facilities and for putting staff at the author's disposal.

REFERENCES

Armstrong, M. T. & Hill, E. G. (1959). The use of gamma-BHC (lindane) and whitewash mixtures to control infestations of *Ephestia elutella* (Hbn.) in warehouses. *J. Sci. Fd Agric.* **7**, 79.

Freeman, J. A. (1948). World foci of infestation and principal channels of dissemination to other points, with suggestions for detection and standards of inspection. *F.A.O. agricultural Studies*, no. 2, Washington, D.C., U.S.A.

Howard, B. H. (1947). The determination of small amounts of hexachloro*cyclo*hexane (benzene hexachloride). *Analyst*, **72**, 427.

Norris, M. V., Vail, E. A. & Averell, P. R. (1954). Colorimetric estimation of malathion residues. *J. Sci. Fd Agric.* **2**, 570.

Parkin, E. A. (1958). A provisional assessment of malathion for stored-product insect control. *J. Sci. Fd Agric.* **6**, 370.

Richards, O. W. & Waloff, N. (1946). The study of a population of *Ephestia elutella* Hübner (Lep. Phycitidae) living in bulk grain. *Trans. R. ent. Soc. Lond.* **97**, 253.

Yeomans, A. H. & Van Leeuwen, E. R. (1954). *Soap, N.Y.*, **30**, 141.

Ann. appl. Biol. (1961), **49**, 588–600
Printed in Great Britain

The depletion of insecticides on sheep fleece

By I. R. HARRISON
Veterinary Research Station, Boots Pure Drug Co. Ltd., Thurgarton, Nottinghamshire
and C. A. JOHNSON
Analytical Development Group, Standards Department, Boots Pure Drug Co. Ltd., Nottingham

(*Received* 24 *February* 1961)

SUMMARY

Sheep have been dipped in various concentrations of aldrin and dieldrin and the rate of loss of insecticide on the wool has been determined using a biological assay method. In one experiment with aldrin, the biological assay method was confirmed by chemical analysis. Depletion is rapid immediately following dipping for a period of 2–4 weeks, after this it is much slower. It has been shown that when dipping is carried out at the normal time, i.e. May to June, the chemical persists for a period of 3–4 months. When sheep are dipped in late July and August, however, the period of protection is much greater. The distribution of insecticide over the fleece has been examined; the area with least pick-up of insecticide is in the tail region where the wool is coarse. Weather and rate of wool growth could not be correlated with rate of insecticide loss.

INTRODUCTION

Since 1954 dieldrin and aldrin have been extensively used throughout Great Britain for the control of sheep blowfly strike. Except for the hill areas sheep are usually dipped 3–6 weeks after shearing, that is, during June and early July. In hill areas clipping and dipping are frequently done at the same time because of the difficulties of shepherding. Sheep dipped with dieldrin at 0·05% in June are normally protected from body strikes for approximately 16 weeks so that one dipping is usually sufficient to cover the season of blowfly activity. Aldrin is slightly less persistent and 12–14 weeks is the average period of protection. Tail strikes can and do occur much earlier than body strikes, particularly if the wool is stained and dirty.

Studies on the depletion of insecticide in the sheep dipping bath (Harrison & Marshall, 1959) have shown that although the dip strength is initially 0·05% and is carefully topped up, a rapid fall in concentration often occurs. With 100 sheep, for example, a drop of concentration from 0·05 to 0·03% is frequently noted but the subsequent protection from the blowfly strike in the field does not appear to be affected.

Very little is known about the rate of loss of these chemicals from the sheep fleece, the emphasis having been on the total period of protection from blowfly strike given by the insecticide.

To study the rate of depletion of these insecticides, accurate biological assay methods for aldrin and dieldrin on wool were needed. It was also necessary to know the 'total protective period' of a sheep dip, that is, the period during which the blowfly larvae are unable to cause a lesion on the sheep.

METHODS

Direct wool assay method

Du Toit & Fiedler (1953) described an assay method for evaluating insecticides used for sheep blowfly control, but this method was found unsatisfactory for the present experiments and it was found that some modifications were necessary. Four

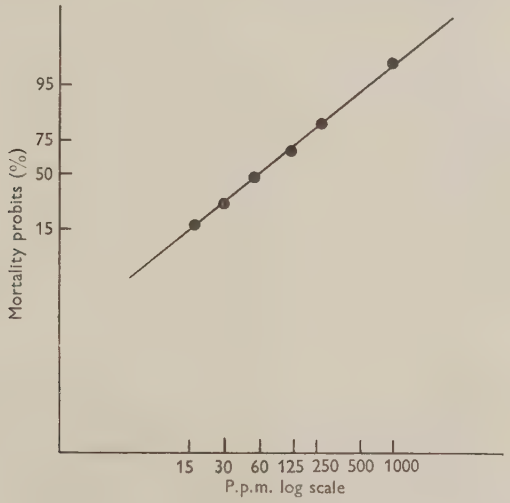


Fig. 1. Aldrin 24 hr. dosage–mortality curve on *Lucilia sericata* larvae.

0·2 g. wool samples from a 10 g. wool sample of a treated sheep were weighed out into small 3 in. × ½ in. diameter tubes. 1 c.c. of sterile sheep serum was pipetted into each tube and this was sufficient to provide food for the larvae without the risk of their becoming trapped. Approximately thirty newly hatched first-instar larvae (± 5) were placed in each tube using a narrow-bladed scalpel. A loose cotton-wool plug was then inserted in each tube. The tubes were kept under a strip light to ensure that the larvae remained in contact with the wool; otherwise the larvae moved up on to the plug and died. After 8 or 24 hr. the tubes were emptied into a Petri dish and the percentage mortality recorded using a light table with a grid. In all cases the results were obtained from four replicates and each experiment was repeated three times.

Standard dosage–mortality curves for aldrin (Fig. 1) and dieldrin (Fig. 2) were plotted for a 24 hr. exposure period. This was done by adding aqueous suspensions of the insecticides prepared from wettable powder formulations to untreated sheep's wool.

Chemical analysis

Various methods for the determination of aldrin on the fleece were considered. Levels to be expected in initial samples were high and thus a number of possible procedures were applicable. It was felt essential, however, to use the same method of analysis throughout, and since levels on later samples were likely to be very low the most sensitive method available was sought. The procedure used was an adaptation of the phenyl azide method for determination of aldrin and dieldrin in crop materials described by O'Donnell, Neal, Weiss, Bann, DeCino & Lau (1954) and O'Donnell, Johnson & Weiss (1955).

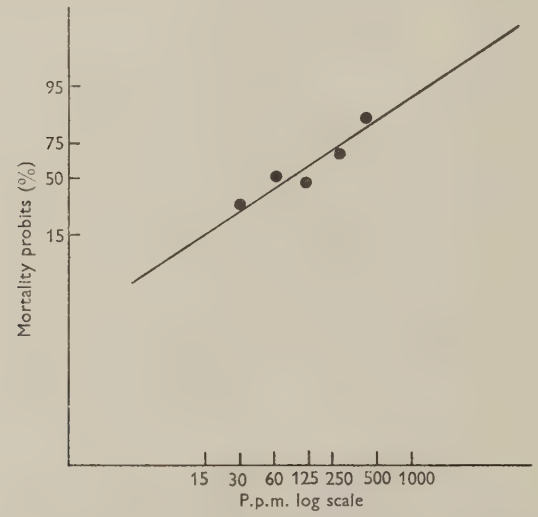


Fig. 2. Dieldrin 24 hr. dosage–mortality curve on *Lucilia sericata* larvae.

As a preliminary, a method was established in which aldrin was removed from the fleece by Soxhlet extractions with petroleum ether (boiling point 66–68°). The extract was concentrated to such a volume that 1 ml. contained the extractive obtained from 1 g. of wool. For the phenyl azide determination the solution was evaporated to remove petroleum ether and was then saponified, aldrin and unsaponifiable matter being extracted from the cooled hydrolysate by extraction with petroleum ether. The ethereal extract was washed with water and then dried and decolorized by shaking with a mixture containing anhydrous sodium sulphate, silica gel, kieselguhr and activated charcoal. After filtration the solution was adjusted to a suitable volume and portions were taken for application of a slightly modified phenyl azide determination. In repeated experiments in which known quantities of aldrin were added to samples of fleece from undipped sheep recoveries of from 90 to 103% were obtained.

In subsequent work it was noted that fleece extracted with petroleum ether for as long as 72 hr. still possessed significant insecticidal properties; a total-solution method

was therefore used in which the whole sample of dipped fleece was hydrolysed, thus ensuring that all the aldrin was determined. Determinations by the solvent extraction and the total-solution methods at various levels showed that a fairly constant amount of aldrin, equivalent to about 0·006%, was not removed by solvent extraction. This amount was also found in wool which had been extracted for a long time. Results recorded in Fig. 5 were obtained by the solvent extraction method and may thus be expected to be a little low; this does not affect the validity of the results in drawing conclusions about the rate of depletion.

The field assessment of blowfly protection

Several methods can be used to assess the total protective period of a sheep blowfly dip. The first and simplest is to dip the sheep during the blowfly season and record the date of the first strike, but it is very unreliable for several reasons. There is much variability in the intensity of blowfly attacks. 1958 was a bad year for strikes, particularly in July and August, when the sheep's fleeces were usually moist and extremely attractive to the fly, whereas the hot dry summer of 1959 resulted in a very low incidence of blowfly strike. An adequate control of the experiment is not possible for if some sheep are left undipped they receive some degree of 'protection' when mixed with dipped sheep and there are large individual differences in susceptibility between sheep.

The application of an attractant substance to treated and control sheep can give better results. Hobson (1936) suggested several chemicals for this purpose, and a mixture of macerated liver and glucose applied to sheep fleece was found to be very effective and was used extensively.

Larval implantation was a more satisfactory method of assessing the total period of protection of a sheep dip (MacLeod, 1937; Stones, 1950). The sheep fleece is parted in the site selected for the implant and the skin after being moistened with a few drops of water is lightly scratched. A cotton-wool plug 2 in. long and $\frac{1}{2}$ in. diameter is applied and pressed down on to the skin. The wool is then closed over the plug and 200–300 first-instar larvae are placed over the plug by means of a pipette. The wool tips are then pulled together and secured with adhesive tape so that the larvae are in the position they would normally be in the fleece. If there is no insecticide present in the fleece the larvae move down and round the cotton-wool plug and develop a strike area on the skin. This technique was used to assess the protection period. The groups of sheep under test were challenged in this way twice and sometimes three times per week until an implant was successful. When an implant was successful it was immediately repeated in a different part of the sheep.

Neither of these methods gives any indication of the rate of depletion of the insecticide on the fleece.

FIELD TRIALS AND LABORATORY EXPERIMENTS

Farm trial 1956

This first trial was with two formulations of aldrin—aldrin miscible liquid and aldrin dispersible powder. It was carried out on a local farm on 21 June and was planned to observe the field protection given by these two formulations during the blowfly season.

440 sheep were dipped in the aldrin dispersible powder and 360 in the miscible liquid preparation. A 300-gallon dipping bath was used and each animal was approximately 20 sec. in the bath and was completely immersed once. The concentration in the dipping bath was checked by taking dip samples at regular intervals for chemical analysis. The sheep in the miscible liquid and dispersible powder dip were marked with distinguishing colours and the subsequent strikes recorded. Nine lambs were

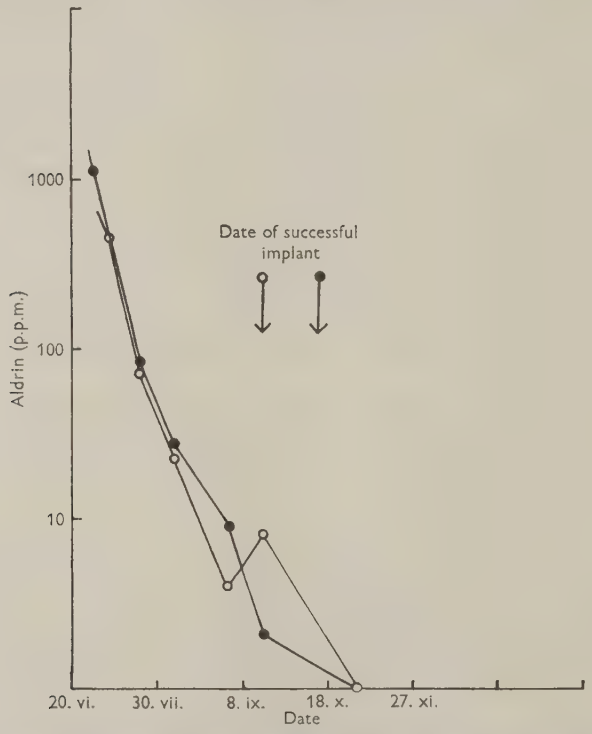


Fig. 3. Depletion of aldrin. Sheep dipped on 21 June 1956. ○, Aldrin dispersible powder, initial strength 0·025 %; ●, aldrin miscible liquid, initial strength 0·05 %; ↓, date of successful larval implant.

selected at known intervals in the dipping series in each treatment so that wool samples could be taken from them for laboratory tests. The ewes and gimmers had been shorn 4 weeks before dipping so that detailed laboratory work was confined to wool samples taken from the lambs. The wool sample in each case was taken from the hip. From the beginning of September three lambs from each treatment were implanted at regular intervals to determine accurately the total period of protection. Records kept of blowfly strike under field conditions in the different flocks showed that only three strikes occurred in late August on ewes, two from the miscible liquid formulation and one from the dispersible powder. They were all confined to a small area above the tail and no serious damage resulted. Fig. 3 shows the results of the laboratory

assays of wool samples taken from the eighteen marked lambs. The wool samples were first assayed separately, but no difference could be detected between samples taken at different stages of the dipping process. Fig. 3 was compiled from the mean of nine wool samples for each treatment. Though the miscible liquid dip concentrations were higher than the dispersible powder this showed only during the first 2 weeks of the experiment. After 10 days' weathering the insecticide concentration on the fleece was

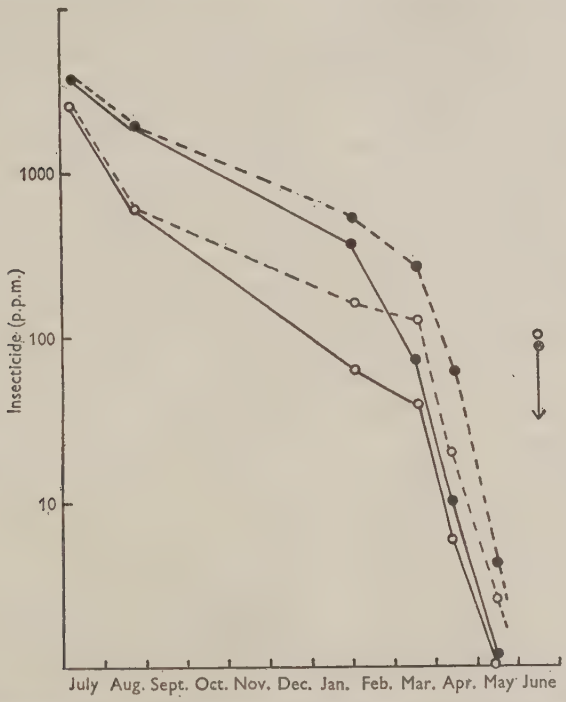


Fig. 4. Depletion of dieldrin and aldrin. Sheep dipped on 12 July 1956 at 0·05 % concentration. Aldrin (—○—), inner 10 mm. of wool; (---○---), outer 10 mm. of wool. Dieldrin (—●—), inner 10 mm. of wool; ---●---, outer 10 mm. of wool; ↓, date of successful larval implant.

very similar in both treatments. During September the six lambs (three from each treatment) were regularly implanted twice per week with blowfly larvae. The dates when the implants were positive are marked on Fig. 3. The aldrin dispersible powder was slightly less persistent than the aldrin miscible liquid but the concentration on the fleece of both formulations fell rapidly during the first month following dipping.

Experimental farm trial 1956

Further work, under carefully controlled conditions, was done on the experimental farm. One breed of sheep (Clun Forest) was used throughout and early-March-born

lambs were used, as wool length and time of clipping in relation to the time of dipping are very important. The lambs are not clipped during their first year, so the wool length could be accurately estimated before the work started. All the dipping was done in a 280-gallon tank and where more than twenty sheep were dipped the dip was pumped out and replaced by a fresh preparation so that the depletion did not affect the experiments. Each lamb was given 20–30 sec. immersion.

Twelve lambs were dipped in 0·05% aldrin and ten lambs in 0·05% dieldrin, both dips prepared from a dispersible powder formulation. The sheep were treated on 12 July 1956, and at weekly intervals after dipping they were implanted with blowfly larvae until a positive result was obtained. The sheep were exposed during the period to natural strike and to increase the chance of its occurrence an attractant paste was applied at weekly intervals. Wool samples taken for laboratory study were divided into two parts for testing, the outer 10 mm. and the inner 10 mm., and the assay results together with the date of positive implant are shown in Fig. 4. At the time of dipping the wool was approximately 60 mm. in length. At the first sampling both parts of the wool contained the same quantity of dieldrin and aldrin. Later samplings showed a reduction in the amount of chemical on the inner part of the sample due to wool growth. Some insecticide moved down the wool grease on the fibre during growth by a process termed 'diffusion' (Du Toit & Fiedler, 1953), but it is not great enough to stabilize the distribution of insecticide along the wool staple. Fig. 4 showed the unusually long protection obtained in this experiment, 10 months instead of the recognized 4 months.

A further dipping experiment on 23 July confirmed these results. Thirty-five lambs were dipped in 0·05% dieldrin dispersible powder and the same tests were carried out during the protective period of the dip.

Experimental farm trials 1957

In the first trial two dips were used, an aldrin miscible liquid and a diazinon miscible liquid. Twelve Clun lambs were dipped in each concentration, the aldrin concentrations being 0·05, 0·025 and 0·012% and the diazinon 0·1, 0·05, 0·025 and 0·0125%.

Table 1. *Number of weeks protection following dipping in diazinon on 22 May* 1957

	Concentration (%)			
	0·0125	0·025	0·05	0·1
	18	13	18	17
	18	17	22	18
	23	18	22	23
Means	20	16	21	19

No laboratory wool testing was done in this trial, but the sheep were implanted with blowfly larvae twice and frequently three times per week. Tables 1 and 2 show the results.

In the diazinon dipping the variation in initial concentrations did not appear to affect the subsequent protective period. Aldrin at 0·05% appeared to be better than the lower concentrations, giving an average of 20 weeks protection with the first strike occurring after 11 weeks, compared with the average of 12 weeks for 0·025% with the first strike occurring after 11 weeks. An average of 13 weeks for the 0·0125% treatment was recorded with the first positive implant in the 9th week.

Table 2. *Number of weeks protection following dipping in aldrin on 22 May 1957*

	Concentration (%)		
	0·0125	0·025	0·05
	9	11	11
	10	11	23
	15	12	23
	19	13	23
Means	13	12	20

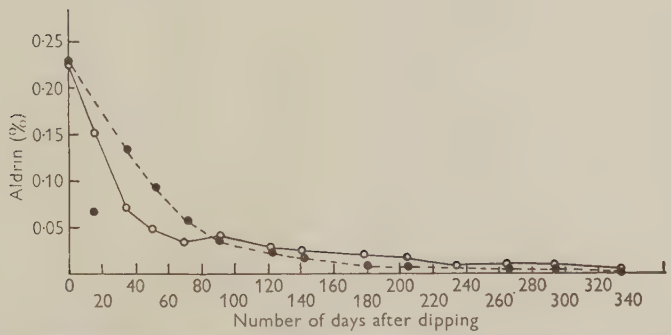


Fig. 5. The depletion of aldrin on sheep fleece dipped on 19 August 1957 at 0·05% concentration. ●, biological assay results (direct wool test); ○, chemical results.

On 19 August 1957, twenty Clun lambs were dipped in aldrin miscible liquid dip at a concentration of 0·05%. The total protective period was followed by fortnightly implants on all the lambs, and in addition to this fourteen wool samples were taken at 14- to 21-day intervals from the hip of each lamb for laboratory testing. The depletion rate of the aldrin on the wool from the time of dipping to the time of blowfly 'strike' was estimated by chemical and biological methods (Fig. 5).

The distribution and persistence of aldrin over the sheep fleece

The uptake of insecticide by the various parts of the sheep fleece varies considerably. In all these trials wool samples were taken from the same area of the hip so that variation between samples from this cause was at a minimum.

On 2 March 1958 one of the lambs died. The fleece was immediately removed and on 17 April detailed assays were carried out on the wool from several areas of the

fleece. Fig. 6 shows how the fleece was subdivided: the areas correspond roughly to the different grades of wool found on a sheep fleece. The finest and most evenly grown wool is found on the shoulders in the area marked 3 and 4. The wool in areas 1 and 2 is similar, but sometimes a little coarser. The wool round the apex of the neck and shoulders (9) varies but is generally not of such good quality. Wool from the areas

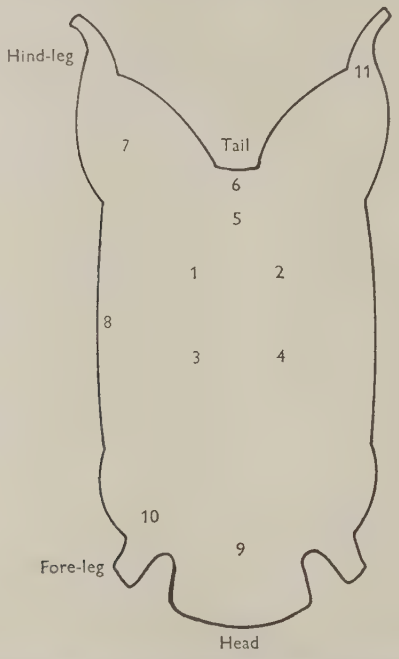


Fig. 6. The distribution of insecticide over the sheep fleece. The eleven regions assayed for aldrin content on 17 April and 2 December 1958.

1, 2, 3, 4, and part 9 is, however, frequently used as one quality. Towards the tail and flanks of the sheep the wool becomes long and coarse, some from area 7 is of fair quality, but area 6 is termed 'breech' wool and it grows in long coarse locks.

The wool length was measured for each of these areas and the wool samples were assayed using the larval method. The wool sample was divided into three portions, an inner 10 mm., an outer 10 mm. and a middle sample of varying length (Table 3).

The results showed the following points of interest:

(*a*) The inner 10 mm. of wool contained very little aldrin in all the sampling areas. This wool represented growth since dipping but it is surprising that more diffusion of insecticide down the fibre had not taken place considering the large amounts present in the outer sample.

(*b*) The fine quality areas of the fleece seem to contain more aldrin than the coarse, poorer quality wool. The samples from areas 6, 7, 8 and 11 contained the least

insecticide; this wool is frequently soiled and continuous rubbing of the wool could account for the loss in these regions.

Blowfly strike is most frequently found in the tail region (areas 5, 6, 7 and 11) and strikes are often found in the neck region (9).

Further series of wool assays were made on 2 December 1958, the fleece having been maintained meantime at room temperature, for in this way it was possible to ascertain whether depletion continues in the absence of wool growth, mechanical rubbing or weathering.

Table 3 shows that there has been an overall slight reduction of insecticide in all parts of the fleece, but it is most pronounced in the coarser wool area.

Table 3. *The variation of insecticide concentration on different parts of the fleece*

		P.p.m. of aldrin					
		17 April 1958			2 December 1958		
Sample position	Total wool length (mm.)	Inner 10 mm.	Middle	Outer 10 mm.	Inner 10 mm.	Middle	Outer 10 mm.
1	138	0	1	166	0	50	10
2	138	0	75	118	0	100	332
3	114	0	276	170	0	163	20
4	119	0	22	276	1	10	1
5	136	13	159	240	163	563	276
6	120	0	28	50	0	23	30
7	138	0	200	22	1	33	10
8	149	0	0	11	0	0	0
9	128	0	42	23	0	0	0
10	124	0	15	33	0	0	1
11	176	0	11	15	0	0	0

After the extraction of aldrin some of the wool samples were checked for residues of chemical using the direct wool test and between 10 and 50 p.p.m. of insecticide was found. The aldrin persisted although the grease and suint were completely removed from the wool fibre so that a much closer bonding of the insecticide to the fibre is indicated. In spite of this, the insecticide is still freely available to the larvae as they move through the extracted wool.

Factors affecting depletion

Some of the factors which may be responsible for the loss of chemical from sheep fleece were examined during the trial. If growth of wool is more rapid during the summer months this could account for the shorter persistence compared with the winter months and the fibre length of the nineteen sheep in the depletion trial was measured.

The wool samples taken from the hip were divided into six equal lots and from each of these ten fibres were taken at random for measurement. Each fibre was held between two fine-pointed forceps and stretched across a piece of black velvet with a centimetre measure along one edge. The stretching was just sufficient to remove the crimp of the fibre. For each sheep, therefore, sixty individual fibres were measured.

The average wool growth for the nineteen sheep from 13 August 1957 to 5 June 1958 is plotted in Fig. 7, which shows that the growth rate for the wool was constant. The more rapid depletion of insecticide during the summer months cannot therefore be attributed to this factor. The lambs were born in mid-March so that for the first year of life the wool growth averaged 10 mm. per month. Supplementary food was fed to the lambs from October onwards throughout the winter.

There was no correlation between rate of depletion (Fig. 5) and rainfall, maximum and minimum temperature, humidity, and sunshine during the period of the experiment.

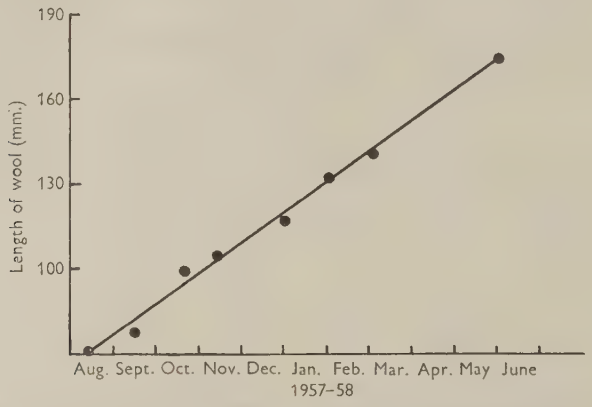


Fig. 7. The wool growth curve for Clun Forest lambs during the period August 1957 to June 1958.

DISCUSSION AND CONCLUSIONS

It was realized from the depletion in the sheep dipping bath that in the field the amount of insecticide each sheep received varies to a large extent but this does not appear to affect appreciably the subsequent blowfly protection. This is clearly demonstrated in the first field trials, and the reasons become apparent when it is realized that the depletion of the insecticide from the wool is very rapid during the first 2–4 weeks. Immediately after dipping the wool contains a large amount—3000 p.p.m. has been demonstrated—but a considerable proportion is lost after 4 weeks. Mechanical detachment probably accounts for much of this, as the insecticide is only loosely attached to the wool. Brushing the fleece against the ground, fences and other sheep in the flock rapidly removes the large particles lodged amongst the wool fibres. After this initial heavy loss the remainder of the insecticide appears to be attached to the wool fibre in one of two ways. The greater part is dissolved in the wool grease, and this is much more stable than loose particles between wool fibres. The depletion from the wool grease is less likely to be a purely mechanical process, but wool growth, movement of wool grease on the fibre and the secretions from the glands around the wool follicle are important. However, tests on dipped wool with all the wool grease removed by extraction with organic solvents showed that some chemical still remains.

When faced with a similar problem with dieldrin, Lipson & Hope (1955) suggested that there might be a chemical combination of the epoxide group with carboxyl and other groups in the wool protein.

Fränkel-Conrat (1944) discussed the action of 1:2-epoxides on proteins, and a reaction between such substances as ethylene oxide or epichlorhydrin and protein carboxyl groups was demonstrated in aqueous solution at room temperature. Capp & Speakman (1949) have published work describing the cross-linking of animal fibres with epoxides, while Alexander, Carter, Earland & Ford (1951) studied the esterification of carboxyl groups in wool with 1:2-epoxides. Lipson & Hope postulated that some such reaction might occur between dieldrin and wool protein, but the results described with the present work indicate that aldrin, which has no epoxy grouping, may show a similar tenacity, so that some other explanation must be sought. The possibility that wool fibres might swell in organic solvents, thus causing insecticide to be trapped between the scales, is not considered very likely since the various solvents used do not appear to have very much effect on wool diameter. The toxic material remaining is recovered when the fibres are completely dissolved but it is apparently also available to larvae moving over the fibre surface.

The study of the depletion curve (Fig. 5) of aldrin goes some way to explain why differences in concentration of dip do not appear greatly to affect the ultimate length of protection. An increase in concentration of the dip may increase the amount on the fleece initially but a large proportion of this may be only loosely attached to the fibre of the wool and will be lost during the 2–4 weeks after dipping.

Du Toit & Fiedler (1953) described the movement of insecticide down the wool fibre to the newly grown wool as 'diffusion'. They listed several insecticides which had this property of diffusion, including aldrin, dieldrin and diazinon. Several experiments have been described where this diffusion has been shown to occur. When the wool staple has been divided into inner and outer 10 mm. portions it is clear that any insecticide present on the inner 10 mm. of wool must have come from the outer part which was present at dipping. This diffusion process must be taking place in the grease on the wool fibre. It is not, however, a straightforward process. If it were a free diffusion it would be expected that, as new growth appeared, the insecticide present on the fibre would be evenly spread over the entire fibre. If depletion by mechanical means were heavy it might be expected that diffusion would not be able to replace the amount of insecticide lost from the outer portion of the wool. In practice, the outer wool always seems to contain more insecticide than the new growth near the skin. Graham (1957) attempted to use this process of diffusion down the fibre for practical purposes. He used light surface sprays of highly concentrated insecticide as a means of controlling body strike. Up to four times the normal concentration of insecticide was found to be necessary to give a reasonable control and this method has never given results to compare with dipping.

The reasons therefore for increased persistence of sheep dip when used late in the season have not been made clear. The portion of the insecticide that is persisting is in the grease on the wool fibre and it seems likely therefore that fluctuations in the secretion of the skin will be very important. Sutton (1933) showed that there was an increase in grease up to shearing time in May and also at the beginning of the winter.

Unfortunately, there are not enough reliable data for Clun Forest sheep to allow a careful correlation to be made.

The authors thank Mr W. M. Brownlie, B.Sc., M.R.C.V.S., D.V.S.M., for his help with this work over the past five years. They are also grateful to the Directors of Boots Pure Drug Co. Ltd. for permission to publish these results.

REFERENCES

Alexander, P., Carter, D., Earland, C. & Ford, O. E. (1951). Esterification of the carboxyl groups in wool. *Biochem. J.* **48**, 629.

Capp, C. W. & Speakman, J. B. (1949). Cross linkings of animal fibres. *J. Soc. Dyers Col.* **65**, 402.

Du Toit, R. & Fiedler, O. G. H. (1953). The protection of sheep against blowfly strike. II. The influence of the length of wool at the time of treatment on the duration of protection. *Onderstepoort J. vet. Res.* **26**, 405.

Fränkel-Conrat, H. (1944). The action of 1,2-epoxides on proteins. *J. biol. Chem.* **154**, 227.

Graham, N. P. H. (1957). Control of fly strike. The use of light surface sprays as a means of controlling body strike. *Aust. Vet. J.* **33**, 137.

Harrison, I. R. & Johnson, C. A. (1958). Persistence of aldrin and dieldrin residues in sheep fleece. *Nature, Lond.* **181**, 1267.

Harrison, I. R. & Marshall, P. G. (1959). The depletion of insecticides in sheep dipping baths. *J. Sci. Fd Agric.* **10**, 568.

Hobson, R. P. (1936). Sheep blowfly investigations. III. Observations on the chemotropism of *Lucilia sericata* Mg. IV. On the chemistry of the fleece with reference to the susceptibility of sheep to blowfly attack. *Ann. appl. Biol.* **23**, 845.

Lipson, M. & Hope, R. J. (1955). *Mothproofing of Wool*, International Wool Textile Research Conference, Australia, Proceedings, E, p. 523: Effect of dieldrin and selected compounds.

MacLeod, J. (1937). The experimental production of cutaneous myiasis of sheep. *Parasitology*, **29**, 526.

O'Donnell, A. E., Neal, M. M., Weiss, F. T., Bann, J. M., DeCino, T. J. & Lau, F. C. (1954). Chemical determination of aldrin in crop materials. *J. Agric. Fd Chem.* **2**, 573.

O'Donnell, A. E., Johnson, H. N. & Weiss, F. T. (1955). Chemical determination of dieldrin in crop materials. *J. Agric. Fd Chem.* **3**, 757.

Stones, L. C. (1950). A comparative method for testing agents used in sheep maggot fly control. *Bull. ent. Res.* **41**, 503.

Sutton, W. G. (1933). Some studies of the yolk in New Zealand wool. II. A study of yolk in the fleece during one year. *J. Text. Inst. Trans.* no. 341.

Ann. appl. Biol. (1961), **49**, 601–615
Printed in Great Britain

Changes in the free amino acids and amides in tomato plants inoculated with tomato spotted wilt virus

By I. W. SELMAN, M. R. BRIERLEY, G. F. PEGG and T. A. HILL
Department of Biological Sciences, Wye College (University of London), near Ashford, Kent

(*Received 24 March* 1961)

SUMMARY

Leaves of tomato plants grown in water culture in growth rooms were inoculated with sap from healthy tomato leaves or with sap containing tomato spotted wilt virus (T.S.W.V.). Changes in the free amino acids and amides of stems and of inoculated leaves, stem growth and infectivity were determined. Samples were analysed 5, 9, 13 and 17 days after inoculation using two-dimensional paper chromatography.

Inoculation with sap from healthy leaves, mixed with celite and sodium sulphite, caused small increases in glutamic acid, asparagine, glycine and serine in the inoculated leaves, and in glutamic and aspartic acids in the stems.

In leaves inoculated with T.S.W.V., glutamine and asparagine increased greatly, reaching maxima at day 13. Both infectivity and amide levels were greater with more concentrated inoculum. The total free amino acids increased in leaves inoculated with T.S.W.V. by 150–180%. Increases were found in α-alanine (day 9, 13 and 17) and in aspartic acid, valine, glycine and the leucines (day 13).

Systemic infection of stems increased total free amino acids by 200–300% and amides by more than 400%. Serine, threonine, phenyl-alanine, proline and the leucines increased at day 9 and reached maxima at day 13. Glutamine, asparagine, glutamic acid, aspartic acid, valine, tyrosine, α-alanine, glycine and γ-amino-butyric acid increased at days 13 and 17.

Changes in total free amino acids in stems and leaves were closely correlated with changes in infectivity.

Five days after inoculation and 3 days before symptoms appeared, the levels of serine and threonine in the stems of plants receiving a dilute inoculum were *less* than those in controls.

It is suggested that accumulation of NH_2 compounds in an infected plant may interfere with virus synthesis; this may explain the observed cyclical changes in infectivity.

Free tyrosine and hydroxylysine were recorded for the first time in leaves and stems of healthy tomato plants and tyrosine and methionine in healthy roots.

INTRODUCTION

The synthesis of virus proteins by plant tissues almost certainly involves changes in their free amino-acid pool,* although such changes may be small and transitory. That changes do occur is now evident from various sources.

Andreae & Thompson (1950) reported that tyrosine and tryptophan were present in healthy potato tubers, but were often undetectable in tubers infected with leaf-roll virus. Allison (1953) could not confirm this, but noted increases in glutamine, glutamic acid and serine in infected tubers.

Diener & Dekker (1954) and Diener (1960) found L-pipecolic acid in peach leaves with Western-X disease, but not in healthy leaves. They considered that the presence of small amounts of pipecolic acid in young, healthy leaves indicated that this acid was a normal metabolite, which accumulated only if the tissues suffered from certain disorders, including arsenic injury. Proline increased in these virus-infected peach leaves. In ring-spot infected *Prunus mahaleb* leaves, the amino acid baikiaine sometimes appeared but infection with mottle leaf or rasp leaf did not much affect the free amino acid or amide content of the leaves.

Fife (1956) found that certain amino acids accumulated in sugar beet leaves infected with curly top and of these arginine increased to the greatest extent. The average increase for all amino acids was more than twofold. Aspartic and glutamic acids were present in greater concentration in diseased leaves than in healthy when sampled in the morning, but in smaller concentration in late afternoon samples.

Laloraya, Govindjee & Rao (1956) reported an increase in asparagine and possibly γ-amino-butyric acid or alanine in leaves of *Carica papaya* infected with 'carica-curl' virus and Govindjee, Laloraya & Rao (1956) found increases in five amino acids in leaves of *Hibiscus esculentus* infected with yellow vein mosaic.

In *Nicotiana tabacum* the amount of glutamine was found to increase within 24 hr. of inoculation with cucumber mosaic virus (Porter & Weinstein, 1957, 1960), but other components were unaffected within 182 hr. In the period 182–238 hr. infection decreased the amounts of amino acids and amides present. Tobacco mosaic virus caused 'a net increase' in serine, glutamine and asparagine in the leaves of tobacco during the early stages of infection (Porter, 1959).

Miczyński (1959) showed that a non-necrotic ring-spot strain of potato virus *X* increased the concentrations of amino acids and amides in still symptomless leaves of White Burley tobacco and in leaves with primary lesions. During the rapid systemic spread of the virus, amino acid concentration was *lower* than in healthy leaves. In tissues of older plants, with the disease well established, a steady increase of aspartic acid and leucines was accompanied by a decrease in glutamic acid.

Both environment and nutrition can induce wide fluctuations in NH_2-compounds in plants, and in the present work virus-induced changes were studied in controlled conditions, as part of studies initiated by Selman & Milne (1961). They reported cyclical changes in infectivity of tomato plants inoculated with tomato spotted wilt virus (T.S.W.V.) and grown in standard conditions.

* Unless otherwise stated, reference to amino acids and amides is to 'concentration' and not 'amount per plant organ'.

Cultural details, maintenance of the virus, methods of inoculation and a new infectivity assay method have already been described (Selman & Milne, 1961). The data from infectivity assays after inoculation with infective sap at two dilutions are directly comparable with the analytical data reported below for Expt. 2.

EXPERIMENTS

Three experiments were made using the tomato cultivar 'Potentate'. In the first two, thirty troughs of nutrient solution were prepared and sixteen plants at the cotyledon stage were planted in each. After 1 week in the growth room (65° F., 15 hr. day, light intensity 450 f.c.) two foliage leaves had expanded and the plants were divided into three groups, receiving different treatments. Infected plants showed symptoms 8 days after inoculation.

Experiment 1

Plants inoculated with virus-infected sap were compared with (*a*) uninoculated controls, and (*b*) controls inoculated with sap from healthy tomato leaves. There were 90 uninoculated control plants and 128 plants in each of the other two groups. Inoculum was prepared by grinding 1 part by weight of leaves with 10 parts by volume of aqueous 0·5 % $Na_2SO_3.7H_2O$ (referred to as dilution 1:10) using celite as abrasive (Selman & Milne, 1961).

Experiment 2

Plants inoculated with infective sap at two dilutions 1:2 and 1:10 were compared with uninoculated controls. There were again 90 uninoculated plants and 128 plants inoculated at each of two dilutions.

Stem heights were recorded for 35 uninoculated plants and for 65 inoculated plants per treatment, and samples were taken for the infectivity assays already reported (Selman & Milne, 1961).

Method of sampling

Duplicate samples of stems (excluding hypocotyls) and inoculated leaves (5 g. fresh weight) were taken for analysis 5, 9, 13 and 17 days after inoculation. The dry-matter contents were determined on each occasion for separate samples taken from 10 to 15 plants per treatment. Diurnal variation in the amino acid content of xylem sap occurs in tomato (Van Die, 1959) and to minimize its effects samples were always taken after about 8 hr. illumination.

Extraction of free amino acids

5 g. samples of fresh leaf or stem were macerated in 30 ml. of chilled 80% ethanol for 5 min. in a top-drive homogenizer at 14,000 r.p.m. The suspension was transferred to a 250 ml. conical flask with 20 ml. of 80% ethanol, stoppered and shaken for 18 hr. at room temperature, then centrifuged (2000–3000 r.p.m.) and the supernatant liquid retained in a refrigerator. The residue was shaken for 24 hr. with 50 ml. of 80% ethanol, centrifuged and the supernatant liquid combined with the first extract. A third extract was obtained in the same way and added to the first two fractions. The residue was dried at 100° C., weighed, and taken for protein hydrolysis.

The alcohol extract was shaken with a mixture of equal volumes of ether and water, and the lower phase which separated was reshaken with ether to give a clear liquid containing the amino acids. This was run through a resin column (15 × 1 cm.) containing 5 g. of 'Zeokarb 225' in the H+ form. The column was washed with distilled water before the amino acids were eluted with 250 ml. of 2N-ammonia. The eluate was evaporated to dryness at 40° C. and the residue dissolved in 1 ml. of 10% iso-propanol. Aliquots of the solution were applied to chromatograms with a micro-pipette. Owing to streaking and other difficulties, certain acids could not be estimated in every sample. In Expt. 1 at least two chromatograms, and in Expt. 2 three chromatograms, were examined for each extract.

Chromatography

An ascending two-dimensional chromatographic technique (Smith, 1958) was employed using Whatman No. 1 (10 × 10 in.) paper in rack tanks at 25° ± 1° C.; 150 ml. of butanol/acetic acid/water (12:3:5) and phenol/water (4:1) were run for 15 hr. as first and second solvents respectively. The first and second solvents were removed from the papers with a forced draught of air at 30° C. for 4 and 12 hr. respectively. After hanging for a further 20 hr. at room temperature the papers were dipped in freshly prepared 0·2% ninhydrin solution in acetone containing 0·1% pyridine. When the solvent had evaporated, the papers were hung for 24 hr. in an incubator at 25° C. with a relative humidity of 40%. Amino acids from experimental material were identified by reference to a map compiled by running pure amino acids and amides in the same conditions.

After development the coloured areas were cut out and eluted separately in 5 or 10 ml. of solvent consisting of an equivolume mixture of M/37·5 Na_2HPO_4–NaH_2PO_4 buffer (pH 7·0) and ethanol. Elution continued for 3 hr. with occasional shaking and the absorbance of the solution determined using 1 cm. cells in a Unicam SP. 600 spectrophotometer calibrated against the eluting solvent. Proline was estimated at 430 mμ, asparagine at 360 mμ, and other acids at 750 mμ. The amount of an acid present in a tissue extract was determined by reference to a standard curve relating absorbance of the ninhydrin derivative to weight of the pure acid chromatographed and estimated in the same conditions.

After cutting out the coloured areas developed with ninhydrin at 25° C., some of the chromatograms were heated at 100° C. for a few minutes but substances developing colour after heating were not estimated quantitatively.

Experiment 3

By the kind co-operation of Prof. M. A. Stahmann and Dr R. Conklin of the University of Wisconsin, a few analyses were made by a method recently developed to separate individual amino acids and amides by direct elution from resin columns (Spackman, Stein & Moore, 1958). Uninoculated plants and plants inoculated with T.S.W.V. (dilution 1:10) were sampled 13 days after inoculation and the vacuum-dried extracts sent to Wisconsin for analysis.

RESULTS

In view of the increase in dry-matter content associated with infection (Table 1), analytical data have been calculated on a dry-matter basis as μg./100 mg. dry weight.

Tables 2–5 show the analytical data for Expts. 1 and 2. Results calculated from the analyses made by Stahmann and Conklin for Expt. 3 are in Table 6. Citrulline, ethanolamine and γ-amino-butyric acid were not estimated in this experiment.

Table 1. *Percentage dry-matter content of leaf and stem samples*

Days after inoculation	Expt. 1						Expt. 2					
	Leaves			Stems			Leaves			Stems		
	U	H	I_{10}	U	H	I_{10}	U	I_{10}	I_2	U	I_{10}	I_2
5	7·9	7·4	7·2	2·7	2·3	3·0	8·0	8·4	8·2	3·9	3·6	3·8
9	7·3	7·1	7·7	3·6	3·4	3·8	8·2	8·8	9·1	4·1	4·8	5·0
13	7·2	6·9	8·5	3·6	3·4	5·3	8·3	9·6	10·0	4·2	5·8	5·9
17	7·1	7·1	8·7	3·9	3·9	5·7	8·4	10·0	10·0	3·9	6·4	5·9

U = uninoculated controls; H = inoculated with sap from healthy leaves; I_{10} = inoculated with T.S.W.V. at dilution 1:10; I_2 = inoculated with T.S.W.V. at dilution 1:2.

Table 2. *The free amino acid and amide content of tomato leaves, μg./100 mg. dry weight (Expt. 1)*

	Control uninoculated				Control inoculated healthy sap, dilution 1:10				Inoculated T.S.W.V., sap dilution 1:10			
Day ...	5	9	13	17	5	9	13	17	5	9	13	17
Amino acid or amide												
α-Alanine	30	83	39	23	40	77	49	16	68	96	63	25
Arginine	ND	.	.	.	.	.	.	.	.	.	.	.
Aspartic acid	55	58	36	25	43	69	21	24	61	66	66	34
Glutamic acid	212	226	193	180	238	252	227	174	238	246	234	196
Glycine	12	24	13	4	21	43	17	2	23	41	50	15
Leucines	.	.	Tr.	.	1	1	Tr.	.	1	2	2	.
Lysine	ND	.	Tr.	.	Tr.	.	4	.	.	.	.	.
Phenyl-alanine	2	1	1	.	.	1	3	.	1	1	9	.
Proline	.	.	.	.	Tr.	.	.	.	.	.	.	.
Serine	37	52	52	12	41	70	58	23	62	76	92	25
Threonine	33	71	26	19	39	61	42	15	62	84	54	23
Tyrosine	.	.	.	.	.	.	.	.	.	.	Tr.	.
Valine	1	5	6	.	1	4	5	.	2	5	10	1
Citrulline	ND	.	Tr.	Tr.	.	.	5	Tr.	.	.	5	Tr.
Ethanolamine	2	7	6	3	3	8	12	2	3	9	5	.
γ-Amino-butyric acid	5	25	25	16	7	12	29	10	11	18	27	13
Asparagine	.	2	9	8	4	13	5	11	5	17	42	31
Glutamine	29	25	24	29	28	47	31	27	29	96	189	112
*β-Alanine	+	ND	+	+	+	+	ND	+	+	ND	ND	+
*Pipecolinic acid	−	ND	−	−	−	−	ND	−	+	ND	+	+
Total	418	579	430	319	467	658	508	304	566	757	848	475

ND = not determined; Tr. = trace present.

* Presence (+) or absence (−) noted after heating chromatogram for colour development.

The methods used for extraction and analysis in Expts. 1 and 2 have certain limitations. For example, losses of ninhydrin-positive substances, especially amides, occurred in the ether used to remove pigments from the alcohol extracts. Some acids, e.g. arginine, need reagents other than ninhydrin for satisfactory colorimetric estimation. Some difficulty was experienced with streaking, especially with glutamic and aspartic acids, but with practice these could be delineated on the chromatogram with remarkable accuracy. α-Alanine, serine, threonine, tyrosine, γ-amino-butyric acid, the leucines, glutamine and asparagine formed discrete spots which could be readily located.

Table 3. *The free amino acid and amide content of tomato stems, μg./100 mg. dry weight (Expt. 1)*

	Control uninoculated				Control inoculated healthy sap, dilution 1:10				Inoculated T.S.W.V., sap dilution 1:10			
Day ...	5	9	13	17	5	9	13	17	5	9	13	17
Amino acid or amide												
α-Alanine	26	17	17	11	21	42	8	5	Tr.	44	84	45
Arginine	.	.	.	.	ND	.	Tr.	.	.	.	Tr.	.
Aspartic acid	144	62	88	75	160	103	77	98	89	52	167	135
Glutamic acid	103	250	234	298	280	345	211	198	177	234	342	356
Glycine	38	35	22	13	24	15	4	16	24	18	67	48
Leucines	.	.	Tr.	.	.	.	.	.	.	3	47	7
Lysine	.	.	.	.	ND	.	.	.	.	.	.	15
Phenyl-alanine	.	.	6	.	.	.	.	.	.	2	55	6
Proline	.	.	.	.	.	.	.	.	.	3	18	12
Serine	57	75	57	71	51	64	53	41	19	112	351	99
Threonine	42	36	9	20	36	48	10	12	11	50	120	57
Tyrosine	.	.	.	.	.	.	Tr.	Tr.	.	Tr.	48	3
Valine	.	.	3	.	.	18	8	.	.	5	47	10
Citrulline	.	.	Tr.	Tr.	ND	.	Tr.	Tr.	.	.	8	.
Ethanolamine	7	12	6	.	.	12	4	.	Tr.	5	15	10
γ-Amino-butyric acid	39	22	38	35	8	26	30	17	Tr.	22	61	42
Asparagine	.	4	13	43	.	26	10	28	.	30	176	119
Glutamine	38	95	65	226	25	106	51	100	12	69	452	480
β-Alanine	+	−	ND	+	−	+	ND	+	−	+	ND	+
Pipecolinic acid	−	−	ND	−	−	−	ND	−	−	+	+	+
Total	494	608	558	792	605	805	466	515	332	649	2058	1444

ND = not determined; Tr. = trace present.

Fig. 1 shows the changes in the total free amino acids and in the amides, glutamine and asparagine, for inoculated leaves (Expts. 1 and 2) relative to uninoculated controls. The changes occurring in the levels of serine, proline, the leucines, phenyl-alanine and threonine in stems are shown graphically in Fig. 2.

Fig. 3 summarizes stem height measurements for Expt. 2. At day 6 the inoculated plants did not differ significantly in height from the controls. At 8 days the I_2 series, but not the I_{10} series, was smaller than the controls ($P = 0.01$), and at 10 days all three treatments differed significantly from one another. Epinasty, leaf distortion and other

Table 4. *The free amino acid and amide content of tomato leaves, μg./100 mg. dry weight (Expt. 2)*

	Control uninoculated				Inoculated T.S.W.V., sap dilution 1:10				Inoculated T.S.W.V., sap dilution 1:2			
Day ...	5	9	13	17	5	9	13	17	5	9	13	17
Amino acid or amide												
α-Alanine	64	62	65	73	71	75	104	96	64	88	87	81
Arginine	7	7	7	7	7	7	6	6	10	7	6	6
Aspartic acid	165	141	175	145	145	277	267	263	198	291	216	248
Glutamic acid	346	382	444	342	366	708	879	616	401	833	840	562
Glycine	24	21	25	20	19	23	31	30	21	31	28	18
Leucines	4	.	4	4	4	7	6	8	7	12	17	9
Lysine	7	1	1	7	6	14	10	12	7	10	8	9
Phenyl-alanine	34	29	29	29	27	30	51	52	33	53	41	36
Proline	.	.	.	.	.	.	.	.	.	.	.	.
Serine	66	48	62	48	58	66	65	59	57	84	59	37
Threonine	49	44	50	70	40	39	62	54	44	46	62	33
Tyrosine	.	.	.	.	.	16	25	20	.	16	20	10
Valine	19	16	17	17	18	30	31	24	21	33	32	24
Citrulline	3	1	4	5	4	2	2	3	5	2	2	2
Ethanolamine	.	.	.	.	4	14	5	12	6	6	.	8
γ-Amino-butyric acid	49	51	47	63	78	65	54	60	70	75	79	69
Asparagine	25	20	15	26	21	40	73	100	20	53	73	63
Glutamine	100	88	90	81	88	178	332	308	110	273	330	259
Total	962	911	1035	937	956	1627	2003	1723	1074	1913	1900	1474

Table 5. *The free amino acid and amide content of tomato stems, μg./100 mg. dry weight (Expt. 2)*

	Control uninoculated				Inoculated T.S.W.V., sap dilution 1:10				Inoculated T.S.W.V., sap dilution 1:2			
Day ...	5	9	13	17	5	9	13	17	5	9	13	17
Amino acid or amide												
α-Alanine	28	34	31	102	21	64	125	113	25	113	113	102
Arginine	39	39	38	46	32	32	55	54	55	67	55	51
Aspartic acid	200	323	248	292	226	232	433	360	252	392	503	367
Glutamic acid	453	1142	962	980	463	1340	1335	1253	553	1374	1294	1081
Glycine	8	10	19	23	8	36	39	39	8	56	29	28
Leucines	8	7	21	79	13	28	142	92	6	83	144	80
Lysine	13	15	10	15	8	18	10	10	6	10	12	17
Phenyl-alanine	51	39	38	97	47	101	156	137	50	137	167	127
Proline	.	.	.	38	.	46	156	154	.	112	156	155
Serine	44	54	52	198	4	136	279	236	39	221	256	229
Threonine	28	35	26	177	26	89	194	171	28	92	139	105
Tyrosine	.	.	.	52	.	16	92	102	.	63	93	91
Valine	28	17	15	43	16	75	162	137	11	94	137	110
Citrulline	5	2	5	10	3	8	10	10	3	6	10	11
Ethanolamine	5	.	.	13	8	.	26	34	6	25	21	33
γ-Amino-butyric acid	72	86	83	86	100	111	121	159	77	188	153	164
Asparagine	57	79	76	175	34	125	239	272	44	169	214	244
Glutamine	116	284	207	572	137	580	1048	1084	146	784	1045	948
Total	1155	2166	1831	2998	1182	3037	4622	4417	1309	3986	4541	3943

symptoms appeared at 8 days and lessened in severity 13 days after inoculation and the growth rate of the stem increased thereafter in both groups of infected plants.

The regressions of the increases in total free amino acids and amides on infectivity (running means from the data of Selman & Milne, 1961) are shown in Fig. 4 for both leaves and stems (data of Expt. 2).

Table 7 shows the total free amino acids and amides in inoculated plants relative to those in the uninoculated for day 13 for Expts. 1–3 which permits some comparison

Table 6. *The free amino acids and amides in leaves and stems sampled 13 days after inoculation and determined by the Moore and Stein method, μg./100 mg. dry weight (Expt. 3)*

(Courtesy of R. Conklin and M. A. Stahmann, University of Wisconsin.)

	Leaves		Stems		
Amino acid or amide	Control uninoculated	Inoculated T.S.W.V., 1:10	Control uninoculated	Inoculated T.S.W.V., 1:10	Roots. Controls uninoculated
α-Alanine	67	73	23	114	28
Arginine	3	5	5	26	2
Aspartic acid	125	237	154	262	38
Glutamic acid	250	428	317	692	149
Glycine	7	26	6	46	7
Histidine	1	8	2	79	6
Hydroxylysine	1	6	.	10	.
Isoleucine	5	12	7	73	6
Leucine	4	8	10	108	11
Lysine	5	6	11	45	9
Methionine	.	.	.	Trace	2
Phenyl-alanine	7	24	6	158	4
Proline	3	14	8	216	18
Serine	45	83	48	With asparagine	44
Threonine	25	53	18	138	16
Tyrosine	3	16	5	128	11
Valine	6	20	7	107	10
Asparagine and glutamine	27	724	83	3660*	70
Total	584	1743	710	5862	431

* With serine.

Table 7. *Relative levels of free amino acids and amides 13 days after inoculation*

(Uninoculated controls = 100.)

	Inoculated leaves		Stems	
Inoculum	Free amino acids	Free amides	Free amino acids	Free amides
T.S.W.V., 1:10				
Expt. 1	155	743	298	805
Expt. 2	172	385	215	454
Expt. 3	183	2690	*c.* 351	*c.* 4410
T.S.W.V., 1:2				
Expt. 2	161	384	212	444
Healthy leaf sap, 1:10				
Expt. 1	118	116	84	78

of the two analytical methods. The data for virus-infected plants showed fair agreement for the free acids but suggested that when large amounts of amides were present, substantial under-estimates were obtained in the first two experiments.

Before discussing treatment effects, the differences in physiological status between uninoculated control plants of Expts. 1 and 2 should be considered. Their dry-matter contents differed (Table 1) and wide variations were found in asparagine, glutamine,

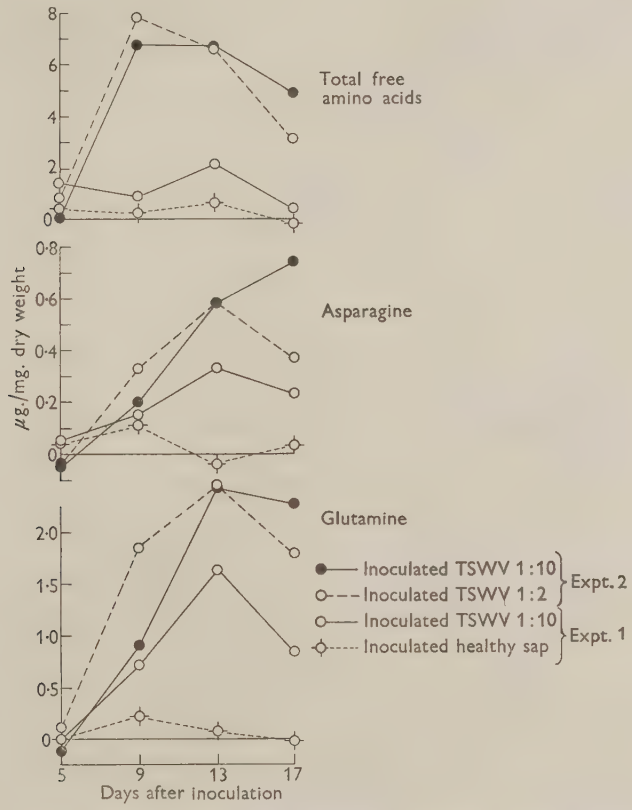


Fig. 1. Changes in free amino acid and amide contents of leaves (inoculated—uninoculated controls).

phenyl-alanine, valine, γ-amino-butyric, aspartic and glutamic acids (Tables 2–5). Margolis (1960) reported that the nitrogen composition of tomato plants receiving a complete nutrient medium, but grown at different times, varied with uncontrolled fluctuations in the environment. Environmental fluctuations in the present work were slight, but there were small differences between plants of the two experiments in stage of development at the time of inoculation. In spite of other differences, serine and threonine in both leaves and stems (days 5 and 9) were little affected and α-alanine

only slightly. For day 13, including the data from column chromatography, the estimates for serine in the uninoculated plants agreed more closely than for any other acid (leaves: 52, 62 and 45; stems: 57, 52 and 48 μg./100 mg. dry weight).

Effects of leaf abrasion. Mechanical inoculation of leaves involving the use of celite is expressly designed to injure living cells. Cell damage might be expected to increase respiration and water loss, both of which could start proteolysis. Small increases were

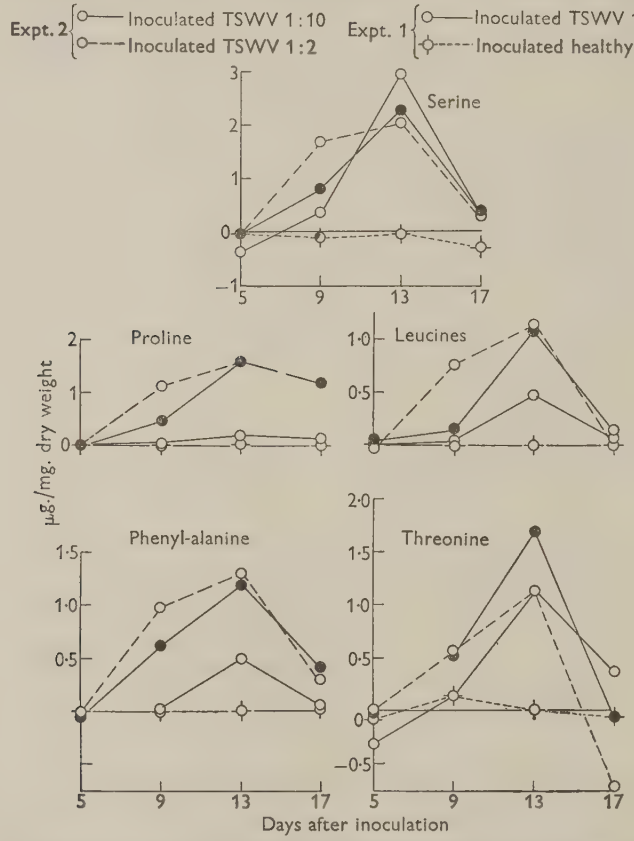


Fig. 2. Changes in free amino acids in stems (inoculated—uninoculated controls).

found in glutamic acid, asparagine, glycine and serine at days 5 and 9 after inoculation with healthy sap (Table 2). At day 5, the dry-matter content of the leaves was decreased by inoculation in both the H and I series (Table 1). Abrasion indirectly increased aspartic and glutamic acids in the stems at day 5 (Table 3). Free amino acids in the tissue may affect susceptibility to infection. Thus, darkening plants for 24 hr. increases their susceptibility to many virus infections and the increase in free amino acids by such treatment is as probable an explanation as those mentioned by Bawden (1956, p. 277). The chemical effects of abrasion merit further study.

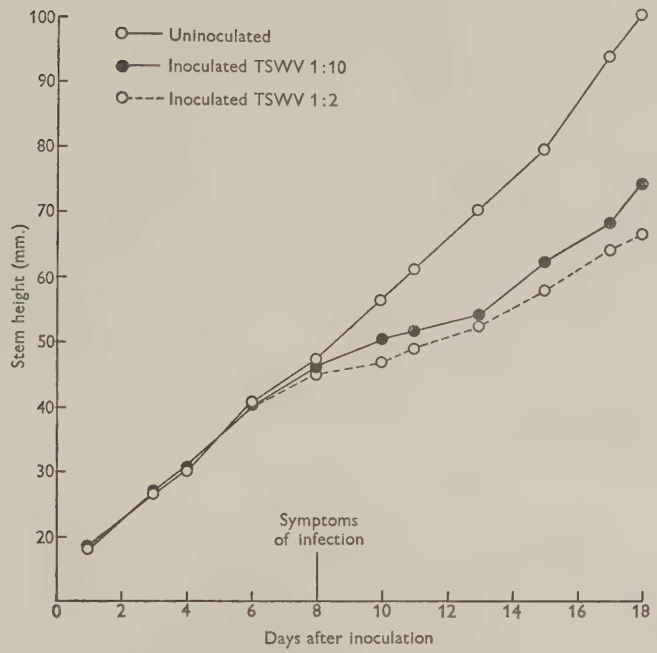


Fig. 3. Stem height and strength of T.S.W.V. inoculum (Expt. 2).

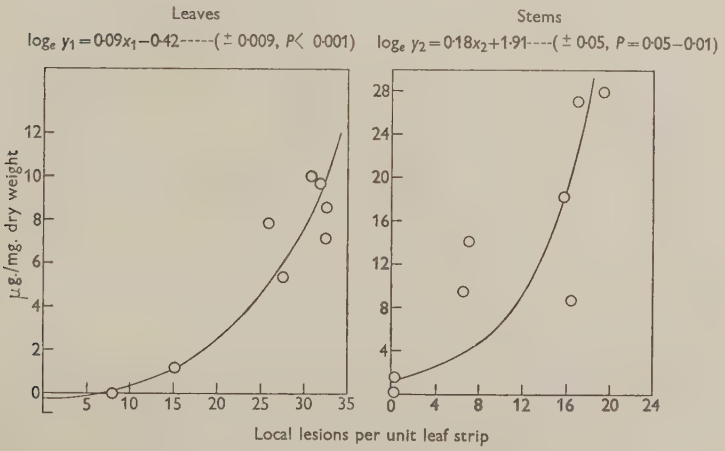


Fig. 4. Regressions of increases in total free amino acids and amides in leaves and stems on infectivity (Expt. 2).

Virus infection in the inoculated leaves greatly increased glutamine and asparagine to maxima usually at day 13 (Fig. 1). With the more concentrated inoculum (Expt. 2) infectivity and the increases in glutamine and total free amino acids were all higher at day 5 than with the weaker inoculum (Fig. 1).

The total free amino acids increased with infection, but the magnitude of the increase differed in the two experiments (Fig. 1). α-Alanine increased in both experiments (day 9, 13 and 17), as did aspartic acid, valine, glycine and leucines (day 13 only).

Virus infection in the stems. Systemic invasion of stems increased the total free amino acids and amides (Tables 3 and 5) and the free acids, serine, threonine, phenylalanine, proline and the leucines increased to a maximum at day 13 and thereafter declined (Fig. 2). The magnitude of the absolute changes in serine with infection of the stem agrees well in both experiments. Glutamine, asparagine, glutamic acid, aspartic acid, valine, tyrosine, α-alanine, glycine and γ-amino-butyric acid showed consistent increases at days 13 and 17.

Citrulline, lysine, histidine and arginine showed no consistent reaction to infection but the determination of these acids was not always satisfactory.

DISCUSSION

The data obtained by Stahmann and Conklin showed that at day 13 every substance estimated in both leaf and stem increased with infection (Table 6). Commoner & Dietz (1952) and Commoner & Nehari (1953) reported that, in tobacco leaf discs infected with tobacco mosaic virus, transitory deficiencies of certain free amino acids occurred when the virus was multiplying rapidly. Smaller deficiencies observed at an earlier stage were thought to be associated with synthesis of virus protein precursors. Evidence for a reduction in the concentration of amino acids during the time of rapid systemic spread of potato virus *X* in tobacco has also been given by Miczyński (1959). We also found that some acids and amides were less in plants 5 days after inoculation with T.S.W.V. than in the uninoculated controls (Tables 3–5). With tobacco mosaic, specific acids have been cited as promoting virus multiplication, viz. glutamic acid (Hirth & Segretain, 1956) and asparagine (Nikiforova, 1958). The decrease in serine and threonine at this early stage of infection possibly indicates their rapid incorporation into virus protein. This observation needs thorough investigation, for chemotherapy designed to check virus synthesis might well be directed at metabolites undergoing such changes during this early stage of infection.

Fig. 4 shows the close correlation of the increases in the total free amino acids and amides of both leaves and stems with infectivity. Extrapolation of the regression line for leaves indicates that when infectivity was low, viz. less than 5 days after inoculation, total acids and amides may have decreased, which would be in agreement with the changes mentioned above for individual acids at day 5. No indication of this effect was seen with systemically infected stems. The decline in the relative amino acid level after the 13th day corresponded with the beginning of the first cyclical fall in infectivity reported earlier (Selman & Milne, 1961).

The incubation period was always 8 days. From the 8th to 13th day in Expt. 2, growth in stem height of the infected plants was severely checked (Fig. 3), but after

13 days some recovery was apparent, and by day 17 the youngest leaves showed only slight symptoms. Stunting therefore may not be caused simply by virus content, as measured by infectivity, or by an accumulation of free amino acids and amides, because both were maximal when infected plants initiated the 'recovery' phase of increased vegetative growth.

In our work the accumulation of amino acids and amides seems to occur as a result of the initial check to growth and presumably to normal protein synthesis, induced by metabolites associated with virus synthesis, but probably not by virus *per se*. The nature of these interfering substances and the reason for their decline in activity after day 13 remain obscure, although as the concentration of free acids increases, some of these could conceivably act as inhibitors of virus multiplication. Thus, for example, histidine and methionine decrease the number of T.M.V. lesions in *Nicotiana glutinosa* (Van Raalte & van der Want, 1952). For tobacco mosaic virus, at least, it is apparently possible for two of the common acids to affect virus production in opposite directions, glutamic to promote and aspartic to inhibit multiplication (Hirth & Segretain, 1956). Examples of antagonism between naturally occurring amino acids have often been observed in nutritional experiments with bacteria and animals (Meister, 1957) and there seems to be no *a priori* reason why similar effects should not occur in the cells of higher plants in relation to protein synthesis.

The full significance of the accumulation of amino acids and amides for the metabolism of the infected plant is not yet clear. In other circumstances, increased levels of free amino N can increase respiration rate (Gregory & Sen, 1937; Wood & Petrie, 1938). Increased respiration rates have often been reported with virus infection and, as Bawden pointed out (p. 287), such increases are not always confined to diseases in which carbohydrates accumulate.

In the equable environment of water culture and a growth room, the response of the tomato to T.S.W.V. leads one to suspect the existence of some kind of 'feed-back' mechanism whereby some of the accumulated metabolites tend to rectify in some measure the abnormal state induced by infection so that growth may be renewed and virus synthesis decline. Under more natural conditions, hot sun or drought acting on an infected plant might lead to conditions under which accumulated metabolites reached toxic levels and killed cells. Necrosis in virus infection is often accompanied by dissolution of starch from neighbouring tissues (Bawden, p. 287), and it is of interest that one of the implicated metabolites, viz. asparagine, can greatly increase the hydrolytic activity of amylase (Hartt, 1934).

In this connexion the findings of Steinberg, Bowling & McMurtrey (1950) and Richards & Coleman (1952) may be relevant. Steinberg *et al.* claimed that the condition of 'frenching' of tobacco leaves may be induced by isoleucine in the tissues, and Richards & Coleman showed that the symptoms of potassium deficiency in barley were associated with accumulation of the diamine putrescine. Certain disease syndromes may evidently be associated with accumulation of NH_2 compounds, and although no such conclusion can be drawn for T.S.W.V. infection in the present work, symptoms of disease were always mild and necrosis rare, whereas in the glasshouse they were more severe.

When the data of Stahmann and Conklin for day 13 (Table 6) are compared with

Tables 2–5 they show clearly that presence or absence of a specific acid in an extract, as indicated by two-dimensional paper chromatography, are relative terms. Mere presence or absence of single NH_2 compounds is unlikely to be the cause of virus disease syndromes.

Tyrosine, methionine and hydroxylysine have not hitherto been reported in the free amino acid pool of healthy tomato plants, although an oxidase, believed to be tyrosinase, was found in tomato plants infected with T.S.W.V. (Best, 1937).

Part of this work was carried out during the tenure of a Research Fellowship awarded to one of us (M. R. B.) by Messrs Fisons Ltd. We are indebted to Prof. M. A. Stahmann for generous permission to include the analyses made at the University of Wisconsin. Sincere thanks are also due to Miss J. R. Brett for technical assistance.

REFERENCES

Allison, R. M. (1953). Effect of leaf roll virus infection on the soluble nitrogen composition of potato tubers. *Nature, Lond.* **171**, 573.

Andreae, W. A. & Thompson, K. L. (1950). Effect of leaf roll virus on the amino acid composition of potato tubers. *Nature, Lond.* **166**, 72.

Bawden, F. C. (1956). *Plant viruses and virus diseases*, 3rd ed. Waltham, Mass: Chronica Botanica Co.

Best, R. J. (1937). On the presence of an 'oxidase' in the juice expressed from tomato plants infected with the virus of tomato spotted wilt. *Aust. J. exp. Biol. med. Sci.* **15**, 191.

Commoner, B. & Dietz, P. M. (1952). Changes in non-protein nitrogen metabolism during tobacco mosaic virus biosynthesis. *J. gen. Physiol.* **35**, 847.

Commoner, B. & Nehari, V. (1953). The effects of tobacco mosaic virus synthesis on the free amino acid and amide composition of the host. *J. gen. Physiol.* **36**, 791.

Diener, T. O. & Dekker, C. A. (1954). Isolation and identification of L-pipecolic acid from Western-X diseased peach leaves. *Phytopathology*, **44**, 643.

Diener, T. O. (1960). Free amino acids and amides in healthy and virus-infected cherry and peach leaves. *Phytopathology*, **50**, 141.

Fife, J. M. (1956). Changes in concentration of amino acid in leaves of sugar beet plants affected with curly top. *J. Amer. Soc. Sugar Beet Technologists*, **9**, 207.

Govindjee, R. V., Laloraya, M. M. & Rao, T. R. (1956). Formation of asparagine and increase in the free amino acid content of virus-infected leaves of *Abelmoschus esculentus*. *Experientia*, **12**, 180.

Gregory, F. G. & Sen, P. K. (1937). Physiological studies in plant nutrition. VI. The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by nitrogen and potassium deficiency. *Ann. Bot., Lond.*, N.S., **1**, 521.

Hartt, C. E. (1934). Some effects of potassium upon the amounts of protein and amino nitrogen, sugar and enzyme activity of sugar cane. *Plant Physiol.* **9**, 453.

Hirth, L. & Segretain, G. (1956). Quelques aspects de la multiplication du virus de la mosaïque de tabac en culture de tissus. *Ann. l'Inst. Pasteur*, **91**, 523.

Laloraya, M. M., Govindjee, R. V. & Rao, T. R. (1956). Increased formation of asparagine in 'Carica-curl' virus-infected leaves. *Experientia*, **12**, 58.

Margolis, D. (1960). The range of free amino acids and amides in tomato plants and the effects of nitrate or ammonium as nutrients. *Contr. Boyce Thompson Inst.* **20**, 425.

Meister, A. (1957). *Biochemistry of the amino acids*, chap. 2. New York: Academic Press.

Miczyński, K. A. (1959). Studies on the free amino acid composition of tobacco plants infected with potato virus *X*. *Acta biol-cracov.* **2**, 23. (*Rev. appl. Mycol.* **40**, 127.)

Nikiforova, G. S. (1958). The sources of nitrogen in the synthesis of protein in tobacco mosaic virus. *Trud. Inst. Genet., Moscow*, **24**, 268. (*Rev. appl. Mycol.* **38**, 226, 1959.)

Porter, C. A. (1959). Biochemistry of plant virus infection. *Advanc. Virus Res.* **6**, 75.

Porter, C. H. & Weinstein, L. H. (1957). Biochemical changes induced by thiouracil in cucumber mosaic virus-infected and non-infected tobacco plants. *Contr. Boyce Thompson Inst.* **19**, 87.

Porter, C. A. & Weinstein, L. H. (1960). Altered biochemical patterns induced in tobacco by cucumber mosaic virus infection, by thiouracil, and by their interaction. *Contr. Boyce Thompson Inst.* **20**, 307.

Richards, F. J. & Coleman, R. G. (1952). Occurrence of putrescine in potassium-deficient barley. *Nature, Lond.* **170**, 460.

Selman, I. W. & Milne, R. G. (1961). A new local lesion assay method for tomato spotted wilt virus, with a note on cyclical changes in infectivity. *Plant Path.* **10**, 100.

Smith, I. (1958). *Chromatographic techniques*. London: Heinemann Medical Books Ltd.

Spackman, D. H., Stein, W. H. & Moore, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Analyt. Chem.* **30**, 411.

Steinberg, R. A., Bowling, J. D. & McMurtrey, J. E. (1950). Accumulation of free amino acids as a chemical basis for morphological symptoms in tobacco manifesting 'frenching' and deficiency symptoms. *Plant Physiol.* **25**, 279.

Van Die, J. (1959). Diurnal rhythm in the amino acid content of xylem exudate from tomato plants bleeding under constant environmental conditions. *Proc. K. Akad. Wet. Amst.* **62**, C, 50.

Van Raalte, M. H. & van der Want, J. P. H. (1952). Effect of amino acids on the number of lesions produced in leaves of *Nicotiana glutinosa* after inoculation with tobacco mosaic virus. *Publ. Lab. Bloembollenonderzoek Lisse*, no. 94, 66.

Wood, J. G. & Petrie, A. H. K. (1938). Studies on the nitrogen metabolism of plants. 2. Interrelations among soluble nitrogen compounds, water and respiration rate. *Ann. Bot., Lond.*, N.S., **2**, 729.

Ann. appl. Biol. (1961), **49**, 616–620
Printed in Great Britain

The effect of paracrinkle virus on the growth of King Edward potato at different temperatures and daylengths

By B. KASSANIS
Rothamsted Experimental Station, Harpenden, Herts.
and W. W. SCHWABE
A.R.C. Unit of Plant Morphogenesis and Nutrition, Wye College, Kent

(*Received* 29 *March* 1961)

SUMMARY

The growth of King Edward potatoes of a stock freed from paracrinkle virus was slightly but consistently superior to that of an infected stock, when grown in controlled environments. Although the leaves of the infected plants showed no evident symptoms, their lower water content suggested incipient N-deficiency. Changes in daylength and night temperature affected virus-free and infected plants similarly. Long days and high night temperature decreased tuber formation, and the higher tuber and total dry weights in short days probably resulted from increased leaf area and reduced stem growth.

INTRODUCTION

All stocks of the potato variety King Edward are infected with potato paracrinkle virus whether or not they show symptoms.

On the assumption that the apical meristems of virus-infected plants may be free from virus, apices were excised from sprouts on tubers and grown in culture media (Kassanis, 1957). In the summer of 1956 one of the plants raised from tissue culture was found to be uninfected and it was propagated by rooting cuttings. The plants grew vigorously but when harvested in October none had any tubers. Failure to set tubers seemed likely to be the result of growth under unsuitable temperature and daylength conditions, the importance of which on growth and tuber formation in the potato has been known since the early experiments of Garner & Allard (1923) and also Roberts & Struckmeyer (1938). In an attempt to get tubers, further cuttings were taken and these were grown under controlled temperatures and daylengths, and compared with similar cuttings from a stock infected with potato paracrinkle virus. Tubers were obtained and the virus-free stock of var. King Edward has since been propagated in the open in Scotland and in Northern Ireland. Some observers have reported that the virus-free stock produced more tubers and more stolons than the commercial stocks. To see whether virus-free and infected plants do differ in this manner a second comparison was made in 1960, when plants raised from tubers were grown in a controlled environment.

METHODS

The plants used in 1956 were raised from stem cuttings struck in a heated glasshouse before they were put in the different environments. Only plants initially of a uniform size were put in the growth chambers (for a description see Schwabe, 1957). The treatments consisted of a factorial combination of two daylengths and two night temperatures: an 8 hr. day (all as daylight) and a 16 hr. day (8 hr. daylight plus 8 hr. incandescent light of low intensity). The day temperature was 20° C. and the two night temperatures were 20° and 13° C. Five virus-free and five infected plants were given each treatment. After 6 weeks in these various conditions, the plants were harvested and their dry and fresh weights, leaf area, water contents, etc., determined. The total leaf area was estimated by finding the fresh weight of discs of unit area, sampled by a cork borer from the fresh leaves, and multiplying by the total leaf fresh weight.

The single set of conditions used in 1960 consisted of a uniform day temperature of 21° C. and a night temperature of 15° C. combined with a 10 hr. photoperiod of 8 hr. daylight and 2 hr. incandescent light. The plants were raised from freshly harvested tubers, whose dormancy was broken by keeping them overnight in an atmosphere of ethylene (0·3 ml. of 'Rindite' per kg. of tubers; Denny, 1945). The terminal 'eye' with the underlying and surrounding tuber tissue was cut off to a piece weighing 2·5 g. and planted in soil in a heated glasshouse. Although sprouting was uneven, there was a clear difference between the times when the virus-free and infected sets sprouted. Most of the virus-free sets started to grow within 3 weeks from planting, whereas less than half the infected sets did so. To minimize this initial difference between the virus-free and infected stocks, the young plants were matched in pairs according to size, ten pairs being used. This arrangement was then taken into account in the statistical analyses of the results. After 8 weeks in the controlled conditions, the plants were harvested and characterized as in the 1956 experiment.

RESULTS

Table 1 shows the significant main effects and interactions obtained in the 1956 factorial experiment.

As was to be expected, daylength had a large effect on most characters. Perhaps surprisingly, plants given only 8 hr. light a day had greater dry weights than the plants given 16 hr. days. Much of this extra dry weight is accounted for by tubers, which with the long-day plants were few and minute. The short-day plants had greater leaf area than the long-day plants but had fewer leaves, and the leaf dry weights were also significantly less. The usual effect on stem length of increased daylength was very obvious, the plants in long days being very straggly; their stems were about twice as long as those of the short-day plants. The effects of a low night temperature on dry weight, tuber weight and leaf area were also clearly evident.

Comparison between the mean results with the virus-free and infected material shows that the virus-free plants had significantly more leaves and larger leaf areas and dry weights.

Table 2 shows the results of the 1960 experiment in which only a short day and an intermediate night temperature were used. To facilitate comparison of the two experiments some of the data obtained in 1956 with the short-day set of plants are also shown in this table. The only treatment comparison is between the virus-free and infected plants, and because of the variability from the erratic sprouting only a few differences attain the conventional significance level. The tuber fresh weights of the

Table 1. *Significant main effects and interactions of daylength, night temperature and paracrinkle virus infection on growth of rooted cuttings of King Edward potatoes*

Character	Daylength (hr.)		Plant stock		Night temperature (°C.)		L.S.D.
	16	8	Virus-free	Infected	13	20	
Leaf number	32·1	25·5	35·0	22·7	—	—*	1·0
Leaf area (cm.²)	409	529	541	396	516	422	16·2
Dry wt. leaves (g.)	1·10	0·90	1·13	0·87	1·09	0·91	0·034
Water content leaves (% dry matter)	1053	1435	1279	1208	—	—*	11·9
Total length of shoot (cm.)	118·5	56·8	104·9	70·5	—	—*	3·62
Tuber number per plant	0·5	2·5†	—	—	—	—	—
Dry weight tubers	0·03	2·52†	—	—	—	—	—
Total dry wt. per plant (g.)	2·72	3·84	3·56	2·99	3·59	2·96	0·14
Ditto virus-free	2·95	4·17	—	—	—	—	0·28
Ditto infected	2·47	3·52	—	—	—	—	
Fresh wt. tubers (g.)	0·20	16·6	—	—*	19·9	13·4	1·32

* Difference not significant.
† Difference too large to require statistical confirmation.

Table 2. *A comparison of potato experiments* 1956 *and* 1960

Character	1956 (Short-day series only)			1960		
	Infected	Virus-free	L.S.D.	Infected	Virus-free	L.S.D.
Dry weight leaves (g.)	0·76	1·06	0·07	0·96	0·97	—*
Dry weight stems	0·247	0·254	—*	0·544	0·401	—*
Dry weight tubers	2·45	2·58	—*	2·18	2·50	N.S.
Dry weight roots	0·054	0·054	—*	0·210	0·215	—*
Total dry weight	3·51	3·84	0·28	3·89	4·07	N.S.
Fresh weight leaves (g.)	11·43	16·17	2·9	15·98	17·06	N.S.
Water content leaves (% dry wt.)	1389	1481	24	1592	1699	89
Fresh weight tubers (g.)	15·99	17·30	N.S.	14·84	17·47	1·9
Water content tubers (% dry wt.)	556	606	38	602	715	N.S.
Number of tubers per plant	2·1	2·7	—*	4·1	3·7	—*
Number of stolons per plant	—	—	—	6·7	8·0	N.S.
Total length of stolons per plant (cm.)	—	—	—	21·3	26·0	N.S.

* Not analysed.

virus-free plants were slightly higher, but apparently not because more tubers were produced. Stolon numbers and total stolon lengths do not differ significantly between virus-free and infected plants. The water contents of leaves and tubers derived from the fresh- and dry-weight determinations are consistently lower in the infected plants in both years.

DISCUSSION

The differences between the virus-free and infected plants indicate that the infection decreases growth even though the leaves of the infected plants show no obvious symptoms. However, this superiority of the virus-free plants is too small to give statistically significant results for all the different characters with the variable plants used in the 1960 experiment. In general the virus-free plants looked the sturdier. In both experiments leaves and tubers of the virus-free plants have greater fresh weights than those of the infected plants, so, as their dry weights do not differ so much, the water contents of the virus-free plants are higher. Lower water contents of the infected plants are reminiscent of the effect of nitrogen shortage (Gregory & Richards, 1929), and effects of virus infections often resemble those of N-deficiency. Paracrinkle virus, even though it causes no obvious leaf symptoms, apparently has this effect on King Edward plants. Our results do not support the idea that stolon and tuber numbers are increased significantly in virus-free plants.

The environment affected the virus-free and infected stocks in much the same way; only the total dry weights per plant show any significant interaction of stock with daylength, the superiority of virus-free over infected plants being greater in short days than in long days.

It might seem unexpected that plants grown in short days should have accumulated more total dry matter than plants in long days which had twice the period of assimilation, although half of this was at a low light intensity. The long-day plants had longer stems and more leaves but a smaller total leaf area, i.e. individually the leaves of the short-day plants were larger. However, they had less dry weight than the leaves of the long-day plants. Clearly less dry matter was retained in these leaves per unit area than in those of the long-day plants, as is borne out also by their considerably higher water contents; they may also have been thinner, but the thicknesses were not measured. Per unit area, the short-day leaves appear to have been the more efficient photosynthetically; for this reason, and also because of reduced stem growth, more material was available for storage in the tubers. These effects on leaf growth resembled those with several other species (Schwabe, 1956).

In general the morphogenic effects of daylength and temperature on the variety King Edward do not differ substantially from those on other varieties recorded by several authors (e.g. Chapman, 1958; Bodlaender, 1960) since the original observations of Garner and Allard. The presence of paracrinkle infection does not alter these responses, but it is slightly detrimental to growth.

REFERENCES

Bodlaender, K. B. A. (1960). The influence of temperature on the development of potatoes. *Jaarb. 1960 Inst. Biol. Schei. Onderzoek Landbouwgewassen, Wageningen*, **112**, 69.

Chapman, H. W. (1958). Tuberization in the potato plant. *Physiol. Plant.* **11**, 215.

Denny, F. E. (1945). Synergistic effects of three chemicals in the treatment of dormant potato tubers to hasten germination. *Contr. Boyce Thompson Inst.* **14**, 1.

Garner, W. W. & Allard, H. A. (1923). Further studies in photoperiodism, the response of the plant to relative lengths of day and night. *J. agric. Res.* **23**, 871.

GREGORY, F. G. & RICHARDS, F. J. (1929). Physiological studies in plant nutrition. I. The effect of manurial deficiency on the respiration and assimilation rate in barley. *Ann. Bot., Lond.*, **43**, 119.

KASSANIS, B. (1957). The use of tissue cultures to produce virus-free clones from infected potato varieties. *Ann. appl. Biol.* **45**, 422.

ROBERTS, R. H. & STRUCKMEYER, B. E. (1938). The effect of temperature and other environmental factors upon the photoperiodic responses of some of the higher plants. *J. agric. Res.* **56**, 633.

SCHWABE, W. W. (1956). Effects of natural and artificial light in arctic latitudes on long- and short-day plants as revealed by growth analysis. *Ann. Bot., Lond.*, N.S., **20**, 588.

SCHWABE, W. W. (1957). Twelve miniature glasshouses with control of temperature and day-length. In *Control of the plant environment*, ed. J. P. Hudson. *Proc. 4th Easter School of Agric. Sci., Nottingham.*

Ann. appl. Biol. (1961), **49**, 621–633
With 1 plate
Printed in Great Britain

Laboratory and field studies on the relation of arabis mosaic virus to its nematode vector *Xiphinema diversicaudatum* (Micoletzky)

By B. D. HARRISON and R. D. WINSLOW
Rothamsted Experimental Station, Harpenden, Herts.

(*Received* 6 *April* 1961)

SUMMARY

Xiphinema diversicaudatum is widely distributed in southern Britain, mainly in heavy soils, but also in the peat soil of the Fens and in medium textured soils in the south-west. Patches of plants infected with arabis mosaic virus (AMV) in a range of crops including some, like celery and marrow, not before known to be hosts, coincide with patches of soil infested with *X. diversicaudatum*. Roots of some plants infested with the nematode carried small galls; not all such plants were infected with, or even susceptible to, AMV. Adult *X. diversicaudatum* transmitted AMV more readily than larvae, but none of 103 nematodes, transferred when moulting, infected healthy plants. *X. diversicaudatum* were still infective after 24 days in moist peat, free from plants.

X. diversicaudatum is common in several districts in soil from old hedgerows but not in the adjacent fields. Hedgerow trees seem to be hosts of the nematode and at least one, elder, is also a host of AMV. In other districts, plants with AMV occur in crops near to infested hedges or isolated trees, and on land taken into cultivation after being derelict. In the Tamar Valley, Devon, *X. diversicaudatum* is common in pastures; white clover in these often contains AMV. Planting such fields with strawberry leads to disease outbreaks.

A few *X. diversicaudatum* occur down to 3 ft. in soil, but most are at 3–9 in. At a site where arable land has reverted to woodland, the nematodes apparently have spread from a hedge into the woodland at an average rate of 1 ft. per year over a period of 75 years. It is suggested that both *X. diversicaudatum* and AMV are common constituents of natural woodland in Britain and that their incidence has decreased since the advent of agriculture.

INTRODUCTION

Arabis mosaic virus (Smith & Markham, 1944) is associated with and presumably the cause of several crop diseases, the most important being raspberry yellow dwarf (Harrison, 1958), strawberry yellow crinkle (Lister, 1958; Jha & Posnette, 1959), a cherry disease of the rasp-leaf type (Cadman, 1960) and rhubarb mosaic (Schade, 1960). It also infects several weed species and is soil-borne (Harrison, 1958). Diseased plants occur in patches in raspberry, strawberry and white clover crops, and the patches coincide with the distribution of a soil-inhabiting dagger nematode (Harrison & Cadman, 1959) identified as *Xiphinema diversicaudatum* (Micoletzky) by Goodey,

Peacock & Pitcher (1960). This nematode transmitted arabis mosaic virus to healthy strawberry and pea plants (Jha & Posnette, 1959; Harrison & Cadman, 1959). We now describe some additional diseases of plants infected with arabis mosaic virus on land infested with *X. diversicaudatum*, some experiments on the transmission of the virus by the nematode, and studies on the ecology of *X. diversicaudatum* in relation to disease outbreaks.

MATERIALS AND METHODS

The sieving method (Harrison & Cadman, 1959) was used to extract *X. diversicaudatum* from all soils except the highly organic black Fen soil, which was not amenable to sieving. Instead, each 100 ml. sample of the Fen soil was broken up in 400 ml. water and the resulting suspension poured into a modified Oostenbrink elutriator, which was then run for 10 min. at a water flow of 1000 ml./min. The outflow was passed through a series of sieves (50-, 100- and 250-mesh per inch), the material collected on the sieves pooled and transferred to paper tissue ('Scotties', Bowater-Scott Corporation Ltd.) on a Baermann funnel. The nematodes in the bottom of the funnel were counted at 2- to 3-day intervals for a week; usually, about half the total count was reached in 3 days. This method gave reproducible results with the Fen soil (e.g. duplicate aliquots of six soil samples gave figures of 38 and 36, 66 and 70, 29 and 35, 7 and 9, 33 and 12, and 9 and 7 *X. diversicaudatum* per 100 ml. soil), but its weakness is that it depends on the mobility of the nematodes: also, the proportion that is extracted of the total *X. diversicaudatum* in the soil is unknown.

In virus-transmission experiments, the nematodes were handled as described by Harrison & Cadman (1959) and added to pots containing 300 ml. sedge peat in which six pea (*Pisum sativum* L. var. Onward) seeds were sown. Four weeks after sowing, the incidence of infection by arabis mosaic virus (AMV) was determined by inoculating sap from the roots and shoots of the pea seedlings to leaves of *Chenopodium amaranticolor* Coste & Reyn. (Cadman & Harrison, 1960). All transmission experiments were made in an insect-proof glasshouse kept at an average temperature of 18° C.

Naturally infected plants were tested by inoculating sap from their shoots to *C. amaranticolor*. The viruses from such plants were identified as AMV by tests with an antiserum prepared against the type strain. Sometimes virus propagated in *Petunia hybrida* Vilm. was partially purified (Harrison & Nixon, 1960) and used for precipitin tests in tubes; sometimes crude sap from systemically infected *C. amaranticolor* leaves was used in tests made by the gel-diffusion method.

RESULTS

I. *Diseases of plants infected with arabis mosaic virus*

A symptom not noted before in raspberry (*Rubus idaeus* L. var. Malling Exploit) infected with AMV is that young infected canes retain their leaves longer in autumn than healthy ones (Pl., fig. 2), so that in November infected areas appear as pools of green in fields of otherwise bare canes.

Previously undescribed diseases were seen in various plants, all found to be infected with AMV. White clover (*Trifolium repens* L.) at several places in Devon and Somerset

showed vein-clearing or chlorotic blotches on many leaves, but a few infected plants were symptomless.

Patches of stunted AMV-infected celery (*Apium dulce* Mill.), the leaves of which showed chlorotic markings along the veins, occurred in a Norfolk crop. The symptoms were reproduced in celery inoculated with AMV in the glasshouse. In the crop, *X. diversicaudatum* was found in soil around the roots of infected plants but not from other parts of the field.

On land infested with *X. diversicaudatum* in Pembrokeshire, stunted marrow (*Cucurbita pepo* L. vars. Harrison's Early Gem and Clucas All Green Bush) plants infected with AMV showed a yellow mottle on their youngest leaves.

On several occasions AMV was isolated from wild elder (*Sambucus nigra* L.) with bright yellow vein-banding on the leaves (Pl., fig. 1). Cadman (1960) also obtained AMV from this species. The symptoms, which are most obvious in spring, disappear in midsummer but may appear again in leaves unfolding in autumn. AMV was also isolated from three elder trees showing leaf mottling but no yellow vein-banding. Elder seedlings, and cuttings from an apparently healthy elder, were difficult to infect with AMV by inoculation of sap, but 1 out of 19 inoculated cuttings developed symptoms somewhat resembling the yellow vein-banding, suggesting that AMV causes this symptom. Elder yellow vein-banding was seen in many counties in the southern half of Britain, from Cardigan, Pembroke and Devon in the west to Suffolk and Hertford in the east.

AMV was also obtained from runner bean (*Phaseolus multiflorus* Willd.) with a systemic distorting mottle, sweet clover (*Melilotus officinalis* Lam.), and from two common weeds, chickweed (*Stellaria media* Vill.) and annual nettle (*Urtica urens* L.). AMV was obtained from box (*Buxus sempervirens* L.) by Mr W. T. Dale of the National Agricultural Advisory Service.

II. *Transmission experiments with* Xiphinema diversicaudatum

When pea, sugar beet and white clover seedlings were grown for a month in soil containing infective *X. diversicaudatum*, more of the pea seedlings became infected than of the other two species, so pea seedlings were used in later transmission tests. Two experiments to investigate the effect of age of pea seedling on the incidence of infection suggested that seedlings first exposed to *X. diversicaudatum* when 1–2 weeks old were slightly more likely than younger or older plants to become infected in the following 4 weeks, but the differences were small. This contrasts with the infection of turnip with tomato black-ring virus, transmitted by the nematode *Longidorus elongatus*, where the probability of infection decreases with increase in age of seedling (Cadman & Harrison, 1960; Harrison, Mowat & Taylor, 1961).

Table 1 shows that, in four out of five experiments, the estimated percentage of adult *X. diversicaudatum* that transmitted AMV was greater than of larvae. Adults of both sexes apparently transmitted with equal facility and the figures for these are pooled in Table 1. Transmission by adults differed considerably between experiments, whereas larvae fed on infected plants in the glasshouse behaved consistently. Other tests showed that the Hampshire population of *X. diversicaudatum*, when newly collected from the field, caused many more infections than the Rothamsted population.

The difference evidently reflects the different environments in which the nematodes developed and not an intrinsic difference in ability to transmit, because after feeding on infected plants in the glasshouse, the two populations transmitted with similar facility.

Table 1. *Transmission of arabis mosaic virus by adult and larval* Xiphinema diversicaudatum

Source of infective nematodes	Percentage nematodes that transmitted*		
	Adult	Larval	Moulting
Expt. 1. Hampshire strawberry field	8 (100)	0 (120)	—
Expt. 2. As Expt. 1, kept 5 months in glasshouse on infected strawberry	33 (66)	5 (48)	0 (27)
Expt. 3. As Expt. 2	1 (80)	5 (80)	0 (14)
Expt. 4. Rothamsted wood, kept 5 months in glasshouse on infected raspberry	20+ (48)	4 (200)	0 (31)
Expt. 5. Rothamsted wood, kept 8 months in glasshouse on infected raspberry	9 (55)	4 (260)	0 (31)

* Percentage (P) of nematodes that transmitted was estimated by using the expression: $P = 100 \times (1 - \sqrt[n]{Q})$, where Q is the proportion of batches of pea seedlings not infected when each batch was infested with n individuals of *X. diversicaudatum*. Figures in parentheses are the numbers of nematodes used in each experiment.

The few larvae found moulting (Pl., fig. 3) when extracted from the soil were put out separately on healthy seedlings in an attempt to see whether the virus is retained through the moult and then transmitted to plants. If it is, the virus could not be carried solely on the distal part of the mouth spear, for this is shed with the skin during the moult. No infections were obtained with moulting nematodes, but it would be premature to conclude that the virus is carried on the parts shed. Only 103 moulting nematodes were tested and the proportion of these that successfully completed the moult is not known. However, several nematodes kept in water completed their moult within a few hours. These facts suggest that, if the virus can be transmitted after moulting, without the nematodes again feeding on infected plants, the probability of newly moulted nematodes transmitting is smaller than that with other larvae from the same population.

To determine how long *X. diversicaudatum* remains infective when denied access to plants, batches of nematodes were added to moist peat at various intervals before sowing pea seed. Table 2 shows that nematodes kept without plants for 24 days at 18° C. transmitted as often as those provided with plants immediately. Larvae and adults both transmitted after 24 days. Jha & Posnette (1961) obtained transmissions with nematodes kept without plants for about 30 days. In our tests, the nematodes kept in peat without plants for 32 days did not transmit, but they may have died. Tests with nematodes in moist sand, from which they are more readily extracted, show that many died in 3 weeks at 20° C.

The procedures of extracting and handling *X. diversicaudatum* may decrease their longevity or infectivity. To determine the persistence of infectivity in a less disturbed population, an organic soil from the site of an outbreak of raspberry yellow dwarf was

sieved through a quarter-inch mesh to remove large root fragments, put in pots and planted in the glasshouse with raspberry varieties Malling Exploit and Malling Jewel (susceptible and immune to AMV respectively). After 8, 11 and 15 months the raspberry plants were removed, the soil again sieved and 300 ml. aliquots used to grow peas or for nematode counts. *X. diversicaudatum* populations survived equally well on the two raspberry varieties, but the population densities in the soil did not increase appreciably until 15 months after the start of the experiment, when they had doubled or trebled. Initially the soil was highly infective and pea seedlings became infected in each of forty 300 ml. aliquots. A few Malling Exploit shoots developed symptoms of

Table 2. *Persistence of arabis mosaic virus in* Xiphinema diversicaudatum *at* 18° C.

	Source and transmission rate of infective nematodes*			
Days without plants	Norfolk raspberry field (20)†	Hampshire strawberry field, kept 5 months in glasshouse on infected strawberry (10)	Rothamsted wood, kept 5 months in glasshouse on infected raspberry (10)	Rothamsted wood, kept 8 months in glasshouse on infected raspberry (9)
0	2/4	1/4	3/6	4/12
4	3/4	1/4	3/6	—
8	1/6	1/4	2/6	—
12	—	—	3/6	4/12
16	—	—	2/6	1/12
24	—	—	—	4/13
32	—	—	—	0/14

* Numerator is the number of batches of pea seedlings that became infected with arabis mosaic virus, denominator is the total number of batches exposed to infection.

† Number of *X. diversicaudatum* per batch of peas.

AMV infection after 5 months and many after 8 months but the Malling Jewel shoots, as expected, remained symptomless throughout. After 8, 11 and 15 months of cropping with Malling Exploit, peas became infected in 10 out of 11, 2 out of 3 and 6 out of 6 aliquots of soil, respectively. The comparable figures for soil cropped with Malling Jewel were 4 out of 12, 0 out of 3 and 0 out of 6. Evidently, the infectivity of populations of *X. diversicaudatum* in soil declines only slowly when an immune plant is grown. Whether AMV persisted for 8 months in small root fragments in the soil planted with Malling Jewel, despite the precautions taken and the prompt removal of weed seedlings, is not known, but the results suggest that some *X. diversicaudatum* may remain infective for up to 8 months.

Schindler (1957), and Schindler & Braun (1957) reported that in the United States *X. diversicaudatum* caused galls on the roots of various plants including rose, strawberry and groundnut. We found root galls on celery (Pl., fig. 4), raspberry (Pl., figs. 5, 6), strawberry and the weed *Stellaria media*. Those on celery were up to 4 mm. in diameter, on raspberry and strawberry about 1 mm. and those on *S. media* smaller. The whole root system of celery seems affected, for AMV-infected plants in a crop infested with *X. diversicaudatum* bore abnormally many fine lateral roots and abnormally few large ones, giving the affected root system a bushy appearance. That root galls occur on Malling Jewel raspberry, which is immune to infection with AMV by

shoot grafting (C. H. Cadman, unpublished), indicates that the virus is not involved in producing the galls. Also, whereas two samples of sap, each from 50 galls on Malling Jewel roots, were inoculated to *Chenopodium amaranticolor* without transmitting AMV, the virus was transmitted from each of two similar lots of galls on Malling Exploit. This difference cannot be ascribed to differing amounts of virus inhibitors in the two varieties, because tobacco necrosis virus was invariably transmitted from the galls on Malling Jewel, although only occasionally from those on Malling Exploit.

III. *Distribution and ecology of* Xiphinema diversicaudatum

General distribution

We have found *X. diversicaudatum* in association with AMV in the counties of Devon, Hampshire, Hertford, Norfolk, Pembroke, Somerset and Suffolk. We have also found the nematode in Bedfordshire, Buckinghamshire, Middlesex, Oxfordshire, Surrey and Sussex. Whether it is equally widely disseminated farther north is unknown.

The proportion of naturally infective nematodes in different populations of *X. diversicaudatum* seems to vary greatly. When pea seedlings were grown for 4 weeks in the glasshouse in unfractionated soils containing known numbers of *X. diversicaudatum*, the proportions of seedlings that became infected suggested that one in six nematodes in a Pembrokeshire soil had transmitted AMV but only 1 in 1800 of those in a Hertfordshire soil. How far the number of nematodes that actually transmitted AMV in these tests differs from the number of infective nematodes is not known.

Many of the sites where *X. diversicaudatum* was found were sampled because plants infected with AMV occurred there. At other sites, *X. diversicaudatum* was found independently of infected plants, and at many of these AMV subsequently proved also to be present. None of the nematode populations that were tested thoroughly proved to be free from AMV, although often the apparent proportion of infective nematodes was small.

The occurrence of *X. diversicaudatum* seems in some way related to soil type. Most infested soils are medium or heavy in texture, and all appear to be types that do not readily dry out. For instance, the water table is high in the organic Fen soils and it was also high in a gravelly soil with a gleyed layer of clay at 18 in. depth. In Devon, where infested soils are of types that drain more freely, the higher rainfall may be a compensating factor; *X. diversicaudatum* has not been found on free-draining soils in the east of Britain. The importance of soil type was particularly well seen in a Suffolk wood at least 200 years old, part of which was on Tertiary pebble beds and part on clay. Elder bushes were common throughout the area, but whereas both the yellow vein-banding disease and the nematode were common on the clay neither was detected on the pebble soil.

Association with hedgerows, woodlands and trees

Elder bushes with yellow vein-banding symptoms are common in Hertfordshire hedges. Soil samples from the root zone of these and nearby symptomless elder nearly always contained *X. diversicaudatum*. Although common in hedges in this district,

X. diversicaudatum was not found 5 yards from the hedges in adjacent arable fields (Table 3). That this distribution results primarily from host preference, not from cultivation, is suggested by counts of nematodes from old grassland adjacent to infested hedges (Table 3, sites 3 and 4). Very few *X. diversicaudatum* were found in the grassland and they were usually restricted to the field margins, into which the roots of hedgerow trees had penetrated. The grassland at site 3 was at least 30 years old and that at site 4 at least a century old, except that it was ploughed in 1942 and reseeded to grass and clover in the following year.

Table 3. *Association of* Xiphinema diversicaudatum *with Hertfordshire hedgerows*

	X. diversicaudatum per 250 ml. soil			
Source of soil	Site 1 (adjacent to arable field)	Site 2 (adjacent to arable field)	Site 3 (adjacent to old grassland)	Site 4 (adjacent to old grassland)
Middle of hedge	58*	30	49	95
Side of hedge	15	21	53	—
5 yards into field	0	0	1	11
10 yards into field	0	0	1	6
20 yards into field	—	—	2	0
30 yards into field	—	—	1	0

* Pea seedlings became infected with arabis mosaic virus when grown in this sample.

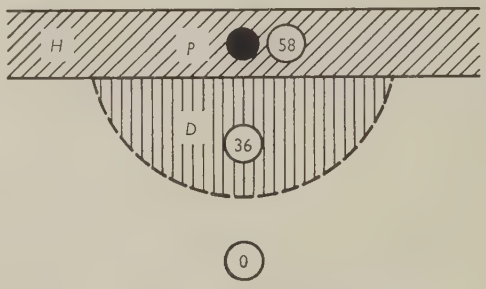
At another site, a row of old ash (*Fraxinus excelsior* L.) and elm (*Ulmus* sp.) trees was bordered on one side by arable land and on the other by old grassland. Over fifty *X. diversicaudatum* per 300 ml. soil were found at 15 yards but very few at 20 yards into the grassland; there were only one or two per 300 ml. soil at 10 yards into the arable land. *X. diversicaudatum* was also restricted to hedges at sites in Pembrokeshire and Sussex. Many *X. diversicaudatum* were found in two cypress (*Cupressus macrocarpa* Gord.) hedges planted as windbreaks.

AMV-infected elder is not uncommon, and other woody hedgerow plants are very probably also hosts of the virus. In a 1-year-old Pembrokeshire strawberry crop, where the rows were 1 yard apart and ran parallel to a hedge containing elder infected with AMV and infested with *X. diversicaudatum*, out of twenty-five plants in similar lengths of the first, second, third and fourth rows from the hedge 16, 4, 2 and 0 respectively were infected. In another crop on the same holding, semicircular patches of diseased plants (Text-fig. 1) occurred next to nematode-infested hedges containing blackthorn (*Prunus spinosa* L.) trees. The patches of infected strawberry plants coincided approximately with the root zone of the blackthorns and with patches of soil infested with *X. diversicaudatum*. On a Hampshire holding, a patch of soil infested with *X. diversicaudatum* and carrying AMV-infected strawberry plants coincided with the root zone of a plum (*Prunus domestica* L.) tree. Here, too, patches of nematode-infested soil and AMV-infected strawberry plants occurred on another piece of land that had recently been brought into cultivation; previously it had been rough grassland with scrub.

All these observations lead to the conclusion that both *X. diversicaudatum* and

AMV have hosts among naturally occurring hedgerow plants and that in many districts they spread from the hedges into crops or occur on land from which woody plants have been removed.

The situation in the Tamar Valley, Devon, appears different. Not only are the hedges (which differ from those referred to above in being earth banks that carry only a few trees and shrubs) poorer sources of *X. diversicaudatum* than adjoining fields, but the nematode seems fairly generally distributed in grassland. For example, *X. diversicaudatum* was found in four out of nine 300 ml. soil samples collected from different grass fields on five different farms in the district. Such grasslands contain much white clover, often infected with AMV, and when the fields are ploughed up and planted with strawberries, patches of plants infected with AMV can appear.



Text-fig. 1. Outbreak of yellow crinkle in a Pembrokeshire strawberry crop. Adjacent to a hedge (*H*) there is an approximately semicircular patch of diseased plants (*D*) apparently centred on a *Prunus spinosa* tree (*P*). The crop was not infected outside this patch. The figures are the numbers of *X. diversicaudatum* per 250 ml. soil that were found at the three sampling points.

Vertical distribution in soil

The vertical distribution of *X. diversicaudatum* in soil is important because of the possibility of disinfesting land by chemicals. Table 4 gives figures for a selection of sites with a range of host plants and soils. In general, the distribution of *X. diversicaudatum* parallels that of the roots, with peak population densities usually at 3–9 in., but there is a tendency for disproportionately many nematodes to occur below the depth of maximum root development. At site A, for instance, few roots occurred at 18–21 in. and at 24–30 in. roots were rare, but some *X. diversicaudatum* occurred down to 33–39 in., the greatest depth sampled. There was evidence of aggregation of nematodes around strawberry plants in the top 6 in. of soil which explains the different distributions at site B, where samples were taken within the row, and at site C, where they were taken between rows. Thus at another site, six 200 ml. samples of soil from 0–6 in. and 6–12 in. depth taken in the plant rows gave counts of 73 and 57 *X. diversicaudatum*, whereas comparable samples from between the rows gave 25 and 50, respectively.

Table 4 also shows that at all sites the proportion of adults increased with depth to reach a constant value at a depth which varied from site to site. We do not know

whether this trend reflects differences in rate of multiplication or mortality at different depths, or vertical migration. Different rates of multiplication or mortality could also explain the unusually small proportion of adults recorded at site D. However, the samples from this site were extracted by the elutriation/Baermann-funnel method, which may extract larvae preferentially.

Table 4. *Vertical distribution of* Xiphinema diversicaudatum *in soil*

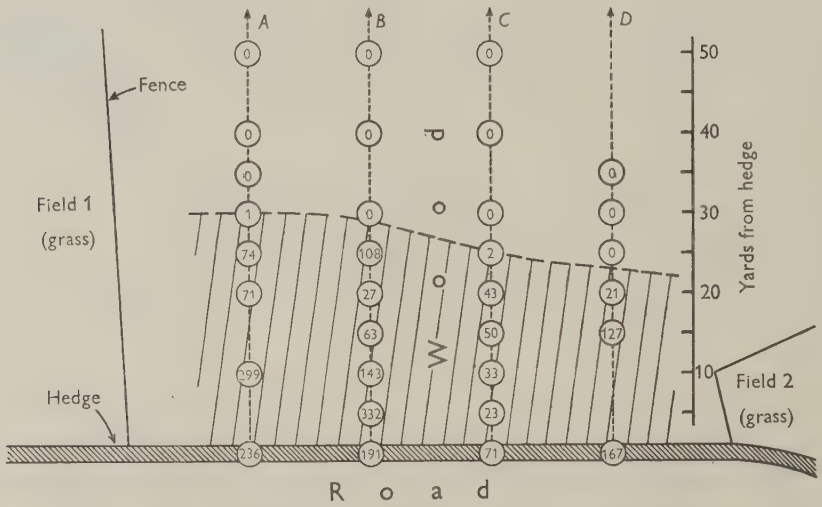
	Site A: woodland (Hertfordshire, February)		Site B: strawberry (Pembrokeshire, June)		Site C: strawberry (Hampshire, October)		Site D: raspberry (Norfolk, November)	
Depth (in.)	No. nematodes*	% adults	No. nematodes	% adults	No. nematodes	% adults	No. nematodes	% adults
0–3	64	16	73	7	3	33	111	1
3–6	241	27	40	36	51	73	—	—
6–9	273	37	8		—	—	204	8
9–12	172	54	5		28		—	—
12–15	122	66	0		—	71	96	
15–18	116	59	—		2		—	
18–21	115		6	50	—	—	0	15
21–24	—	54	—		—	—	2	
24–30	19		2		—	—	0	
33–39	4		0		—	—	0	
Soil type ...	Clay loam top soil with stiff clay below 24 in.		Silty loam throughout		Gravel top soil with gleyed clay below 18 in.		Black organic soil throughout: water-logged below 30 in.	

* Number of *X. diversicaudatum* per 300 ml. soil. Nematodes were extracted by the sieving method, except those from Site D, which were extracted by the Oostenbrink elutriator/Baermann-funnel method.

Rate of spread of Xiphinema diversicaudatum *in uncultivated land*

Part of Geescroft field on Rothamsted Farm was used almost continuously for experiments with field beans from 1852, when records begin, to 1878. It had then become so badly infested with weeds that attempts to control these by fallowing or by growing barley undersown with clover were only partly successful, and in 1886 the area was allowed to revert to natural vegetation. It is now a woodland with a continuous canopy formed by large trees (see Brenchley & Adam, 1915). One side of the area borders a road from which it was originally separated by a hedge containing woody plants. An approximately rectangular area next to the road is now densely infested with *X. diversicaudatum*, but the nematode was not found elsewhere in the wood (Text-fig. 2). Mr B. W. Avery of the Soil Survey of England and Wales could find no obvious differences between the soils of the infested and non-infested areas. We think the infested area is that into which *X. diversicaudatum* has spread from the old hedge since 1886. Nematode numbers in old grassland (Text-fig. 2, field 1), which also has a hedge bordering the road, and is separated from Geescroft Wood only by a wire fence, are consistent with this conclusion. The hedge contains many *X. diversicaudatum*, whereas the few in the grassland are restricted to the zone infested with roots of the hedgerow trees (Table 3, site 4). These results suggest that, given suitable hosts, the nematodes spread out from infested hedgerows: they do not support the

idea that too few nematodes survive in cultivated fields to be detected, these few multiplying when suitable hosts are grown. Assuming that *X. diversicaudatum* occurred up to 5 yards from the hedge in 1886, the rate of spread at the west end of the road hedge is 25 yards in 75 years, or 1 ft. per year. At the east end, where the soil is usually drier and seems less favourable for *X. diversicaudatum*, the population density being generally lower, the apparent rate of spread is about 9 in. a year.



Text-fig. 2. Diagram showing the present distribution of *X. diversicaudatum* in Geescroft Wood. The figures are the numbers of nematodes per 300 ml. soil that were found at the sampling points in the wood. *A*, *B*, *C* and *D* are the transects referred to in Table 5. A broken line marks the boundary of the infested area.

Table 5. *Incidence of* Xiphinema diversicaudatum *near the edge of an infested area in Geescroft Wood*

Yards from road hedge	Transect*			
	A	*B*	*C*	*D*
20	71†	27	43	12
21	—	—	—	46
22	—	—	—	2
23	—	—	78	25
24	—	—	24	0
25	74	108	13	0
26	—	200	0	—
27	—	85	0	—
28	62	108	0	—
29	72	25	0	—
30	7	0	0	0
31	0	—	—	—
32	0	—	—	—

* The positions of transects *A–D* are shown in Text-fig. 2.

† Number of *X. diversicaudatum* per 300 ml. soil: all samples were collected at 3–6 in. depth.

The boundary between uninfested land and the area infested with *X. diversicaudatum* is sharp (Table 5), and the general population density is often attained only 2 yards inside the infested area. According to the estimates given above, 2 yards of spread takes about 6 years, suggesting that the population density increases from one to a hundred nematodes per 300 ml. soil in this period, i.e. approximately a doubling per year. How much of this increase comes from nematodes migrating from the previously infested area and how much from reproduction of the migrants is unknown.

DISCUSSION

The study of *X. diversicaudatum* as a vector of AMV can be split into three complementary parts: (1) the manner of transmission, which determines the potentiality of the nematode as a vector; (2) its distribution and ecology; (3) its behaviour in soil, particularly its feeding behaviour, and the effect of environmental conditions on this.

Little can yet be said about the manner of transmission. The form of the root galls suggests that *X. diversicaudatum* often feeds on roots just behind the growing point, but what proportion of the feeding punctures occurs here is unknown. Jha & Posnette (1961) showed that non-infective nematodes can acquire the virus when placed on infected plants for a day and that nematodes raised on infected plants can transmit the virus to healthy plants in a period of 3 days. Their figure of about 30 days' persistence of AMV in nematodes kept without plants is similar to ours and to that for the serologically related grapevine fanleaf virus (Cadman, Dias & Harrison, 1960) in *Xiphinema index* (Raski & Hewitt, 1960). Our results show that, at 18° C., the nematodes were as infective after 24 days without plants as they were initially, and, at the lower temperatures of field soil, infectivity would presumably be retained for longer. By contrast, the infectivity of plant sap containing AMV falls to less than a thousandth in 2 weeks at 18° C. and may be lost completely (Harrison, 1958). Whether the virus is stabilized in the nematode because it multiplies there, or for some other reason, is a subject for further experiment, but our results on the decline of infectivity of *X. diversicaudatum* populations kept on an immune plant suggest that even if the virus does multiply in the nematodes, transmission through their eggs cannot be common, if it occurs at all.

Our observations give some idea of the distribution and ecology of *X. diversicaudatum* in southern Britain. In only two of the districts studied, the early strawberry-growing districts of Devon and Hampshire, do hedgerows seem unimportant as reservoirs of AMV and *X. diversicaudatum*. Both these districts owe their horticultural importance very largely to their mild climate, which perhaps enables the nematode to thrive in soils that would be unfavourable elsewhere. Nearly all the highest population densities of *X. diversicaudatum* were recorded in the root zones of woody perennial plants, which seem the most suitable hosts. However, many kinds of herbaceous plants were found naturally infected with AMV and root galls were found on several of them, indicating that *X. diversicaudatum* is highly polyphagous, in contrast to the closely related species *X. index*, which has been found only on woody plants such as grapevine and fig (Thorne & Allen, 1950; Raski & Radewald, 1958).

Most nematodes important in agriculture seem to have increased in prevalence as a result of cultural practices but *X. diversicaudatum* has probably decreased. On the heavier soils, which are favourable to *X. diversicaudatum*, the climax dominant species are trees (Tansley, 1939) and, as there is no reason to think that the nematode has been introduced to Britain recently, it was probably widespread in the native forests before the advent of farming. In many parts of the country, the efforts of the grazier and the ploughman since Saxon times seem to have pushed back the nematode, and therefore AMV, to the hedges.

Short of chemical control measures, which are now being studied, or use of immune varieties such as Malling Jewel raspberry, there is little possibility of avoiding disease in crops susceptible to AMV on land infested with infective *X. diversicaudatum*. There are, however, a few suggestions from our work which may help to avoid outbreaks of AMV in raspberry and strawberry. It is especially important that planting stocks should not be infected, and these would be most safely propagated on old arable land away from hedges in districts where *X. diversicaudatum* does not occur or where it is confined to the hedgerows. Strawberry runners should not be transplanted with a trowelful of soil, a practice of some growers. A chemical nematicidal dip for runners and canes would be valuable. Finally, where trees are essential for shelter, conifers should be planted when possible, because although these may be hosts of *X. diversicaudatum* there is no evidence that they are hosts of the virus.

We are indebted to those mentioned in the text, and to J. E. E. Jenkins, H. G. Morgan and P. Wiggell of the National Agricultural Advisory Service at Starcross, Devon, for their co-operation; also to Miss J. Hillier for technical assistance.

REFERENCES

Brenchley, W. E. & Adam, H. (1915). Recolonisation of cultivated land allowed to revert to natural conditions. *J. Ecol.* **3**, 193.

Cadman, C. H. (1960). Studies on the relationship between soil-borne viruses of the ringspot type occurring in Britain and Continental Europe. *Virology*, **11**, 653.

Cadman, C. H., Dias, H. F. & Harrison, B. D. (1960). Sap-transmissible viruses associated with diseases of grape vines in Europe and North America. *Nature, Lond.* **187**, 577.

Cadman, C. H. & Harrison, B. D. (1960). Studies on the behavior in soils of tomato black ring, raspberry ringspot, and arabis mosaic viruses. *Virology*, **10**, 1.

Goodey, J. B., Peacock, F. C. & Pitcher, R. S. (1960). A redescription of *Xiphinema diversicaudatum* (Micoletzky, 1923 & 1927) Thorne, 1939 and observations on its larval stages. *Nematologica*, **5**, 127.

Harrison, B. D. (1958). Raspberry yellow dwarf, a soil-borne virus. *Ann. appl. Biol.* **46**, 221.

Harrison, B. D. & Cadman, C. H. (1959). Role of a dagger nematode (*Xiphinema* sp.) in outbreaks of plant diseases caused by arabis mosaic virus. *Nature, Lond.* **184**, 1624.

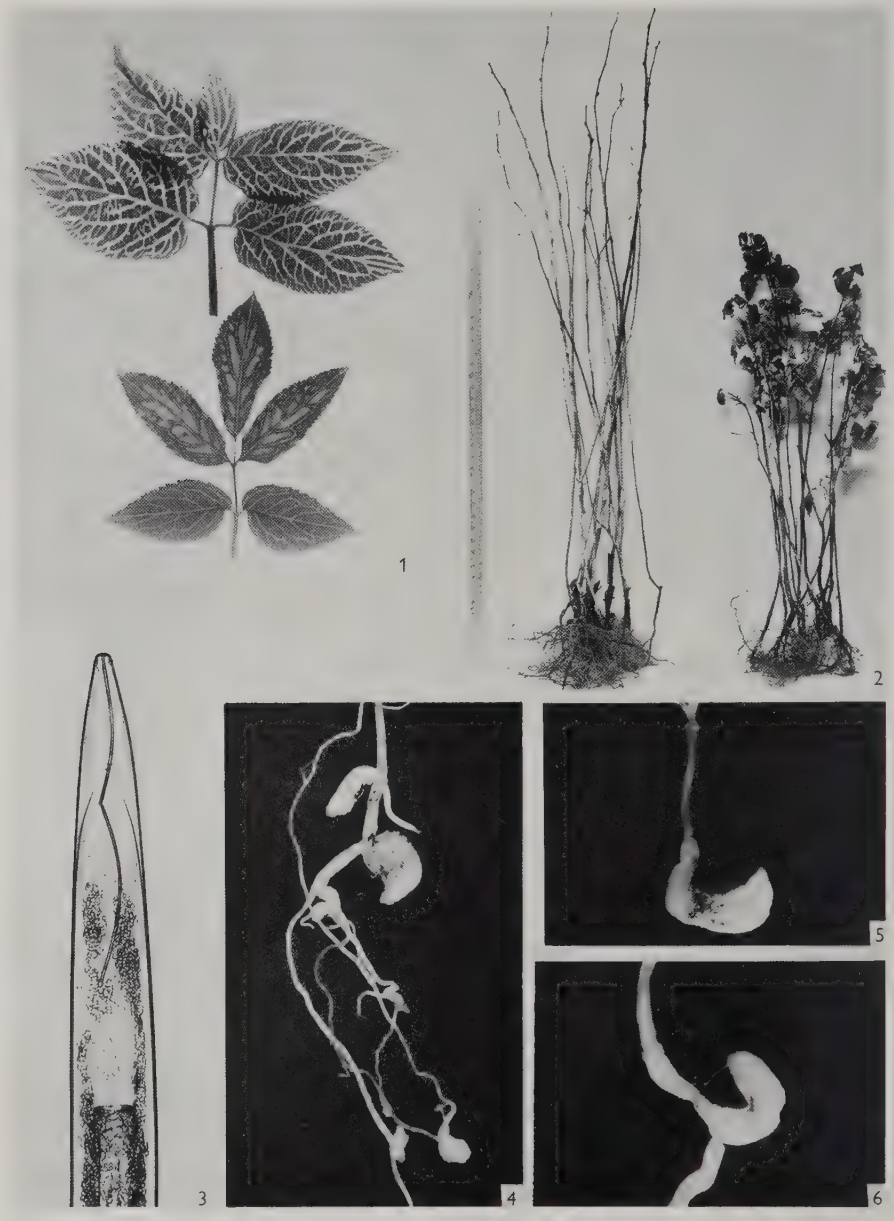
Harrison, B. D., Mowat, W. P. & Taylor, C. E. (1961). Transmission of a strain of tomato black ring virus by *Longidorus elongatus* (Nematoda). *Virology*, **14**, 480.

Harrison, B. D. & Nixon, H. L. (1960). Purification and electron microscopy of three soil-borne plant viruses. *Virology*, **12**, 104.

Jha, A. & Posnette, A. F. (1959). Transmission of a virus to strawberry plants by a nematode (*Xiphinema* sp.). *Nature, Lond.* **184**, 962.

Jha, A. & Posnette, A. F. (1961). Transmission of arabis mosaic virus by the nematode *Xiphinema diversicaudatum* (Micol.). *Virology*, **13**, 119.

Lister, R. M. (1958). Soil-borne virus diseases in strawberry. *Plant Pathology*, **7**, 92.


1
2
3
4
5
6

Raski, D. J. & Hewitt, W. B. (1960). Experiments with *Xiphinema index* as a vector of fanleaf of grapevines. *Nematologica*, **5**, 166.

Raski, D. J. & Radewald, J. D. (1958). Reproduction and symptomatology of certain ectoparasitic nematodes on roots of Thompson seedless grape. *Plant Dis. Reptr*, **42**, 941.

Schade, C. (1960). Untersuchungen zur serologischen Verwandtschaft des Rhabarbermosaikvirus mit dem Gelbverzwergungsvirus der Himbeere (raspberry yellow dwarf virus). *Phytopath. Z.* **37**, 422.

Schindler, A. F. (1957). Parasitism and pathogenicity of *Xiphinema diversicaudatum*, an ectoparasitic nematode. *Nematologica*, **2**, 25.

Schindler, A. F. & Braun, A. J. (1957). Pathogenicity of an ectoparasitic nematode, *Xiphinema diversicaudatum*, on strawberries. *Nematologica*, **2**, 91.

Smith, K. M. & Markham, R. (1944). Two new viruses affecting tobacco and other plants. *Phytopathology*, **34**, 324.

Tansley, A. G. (1939). *The British Islands and their vegetation*. Cambridge University Press.

Thorne, G. & Allen, M. W. (1950). *Paratylenchus hamatus* n.sp. and *Xiphinema index* n.sp., two nematodes associated with fig roots, with a note on *Paratylenchus anceps* Cobb. *Proc. Helm. Soc. Wash.* **17**, 27.

EXPLANATION OF PLATE

(Photographs by Mr F. D. Cowland)

Fig. 1. Two leaves from an elder tree infected with arabis mosaic virus. The upper leaf shows yellow vein-banding.

Fig. 2. Plants of Malling Exploit raspberry lifted in November. Left, healthy; right, infected with arabis mosaic virus. The scale is 1 m. long.

Fig. 3. Photomicrograph of head end of moulting *X. diversicaudatum*. The moulting nematodes used in the transmission experiments mostly had this appearance.

Fig. 4. Root galls on celery from crop infested with *X. diversicaudatum*.

Fig. 5. Gall on root of Malling Jewel raspberry infested with *X. diversicaudatum* in the glasshouse.

Fig. 6. Gall on root of Malling Exploit raspberry infested with *X. diversicaudatum* in the glasshouse.

Ann. appl. Biol. (1961), **49**, 634–644
With 1 plate
Printed in Great Britain

The development of filter-passing organisms in *Corynebacterium fascians* cultures

By MARGARET S. LACEY
Bacteriological Laboratory, Imperial College of Science and Technology, London

(*Received* 28 *March* 1961)

SUMMARY

Viable filter-passing particles have been obtained from gall-tissue cultures of *Corynebacterium fascians*. On gelatine media filtrates of the cultures first produced a faint clouding of the liquefied gelatine; this was due to masses of granules of very variable size. In some cultures further development took place, the next phase being the appearance of diplococci. Sometimes the coccal stage persisted but occasionally complete regeneration of the normal *C. fascians* type rod finally ensued. This sequence of growth follows the pattern designated as the 'L phase', occurring in the life-history of certain bacteria. Growth of L forms probably occurs in *Agrobacterium tumefaciens* also.

INTRODUCTION

During studies on *Corynebacterium fascians*, the causal organism of leafy-galls on sweet-peas and many other host plants, many attempts were made to obtain an active substance from culture or gall extracts which would stimulate abnormal plant growth in the absence of living organisms. Occasionally, definite galls developed on sweet-pea seedlings treated with these extracts, but as *C. fascians* colonies always developed on isolation plates made from the galled tissue it was assumed that a few organisms had survived in the supposedly sterile extracts. Then, in one experiment, three out of six sweet-pea seedlings treated with a culture filtrate obtained by passage through a fritted glass filter developed galls. Although *C. fascians* organisms were isolated from these galls, culture media inoculated with part of the filtrate remained sterile throughout the experiment. The possibility that the filtrate contained filter-passing forms of *C. fascians* was then considered. There have been numerous reports of the occurrence of filterable elements in bacterial cultures, particularly of species pathogenic to man, but for some time considerable doubt was thrown on the existence of such elements, particularly as repetitions of a successful experiment frequently gave negative results. However, the accumulation of evidence for their existence is now sufficient to convince most workers and it is generally considered that filter-passing elements are a phase in the development of L forms of bacteria. Kleinberger-Nobel (1951), who first described a growth which she designated by the letter L, of soft cytoplasmic elements, globules and minute granules in cultures of *Streptobacillus moniliformis*, says: 'the L phase... is here regarded as a phase of bacteria which produces a large number of small filterable viable elements'. She considers that 'the L cycle may be regarded as

a process of regeneration in bacteria probably equivalent to a sexual process in higher organisms'.

The abnormal bacterial forms such as filaments, irregular-shaped rods with bulbous ends or with granular contents, coccal forms and the disintegration of rods leaving chains of granules which occur in cultures of *C. fascians* grown on certain media (Lacey, 1955) are very similar to the pleomorphic forms seen in cultures of *Streptobacillus moniliformis*, *Proteus* sp., etc., where they occur as the preliminary to the development of the L phase (Dienes & Weinberger, 1951). This paper describes experiments which demonstrate the existence of the L phase and the development of filter-passing elements in *C. fascians* cultures.

METHODS AND MATERIALS

Culture media

(1) *Media for the production of the L phase in* Corynebacterium fascians *cultures*

(*a*) As viable filter-passing particles were first obtained from a culture of sweet-pea galls in water, variations of this simple medium were used in most of the experiments. Sweet-pea seedlings grown under sterile conditions in sand culture were inoculated with *C. fascians*. The resultant galls were removed aseptically and dropped into flasks of sterile tap water (4–8 galls, depending on their size, in 50 ml. of water). The best results were obtained when the suspension was not subjected to sterilization, the *C. fascians* organisms present on the galls acting as the inoculum. Experiments in which a short heating of the medium of 5–10 min. at 10 lb. pressure in an autoclave was followed by re-inoculation with a *C. fascians* culture were either negative or the filter-passing particles were produced more slowly and in fewer numbers.

(*b*) A naturally occurring leafy-gall was crushed in water, then filtered free from bacteria through a Seitz filter. Inoculation of this medium with *C. fascians* gave positive results, but a medium composed of pieces of the gall placed in a flask of tap water and sterilized in the autoclave was ineffective.

(*c*) *Brussels sprouts medium.* Media (*a*) and (*b*) were unsatisfactory for the examination of a number of different strains of *C. fascians* for the production of filter-passing particles, as (*a*) was infected with the *C. fascians* strain used for the sweet-pea gall production and (*b*) might already contain filter-passing elements from the strain infecting the gall. An effective medium using Brussels sprouts was found; made as follows: The outer leaves and the central cores were removed from 1 lb. of sprouts. The rest of the tissue was minced up, put in 500 ml. of rain water and extracted for 24 hr. After clearing through filter-paper the fluid was passed through Berkefeld filter candles (no. 5) and distributed in sterile flasks, 50 ml. in each flask. The medium was tested and found to be free from filter-passing particles. Positive results were obtained by the use of this medium but dilutions of it, and also a medium composed of autoclaved chopped-up tissue in water, were ineffective.

(*d*) *Penicillin in nutrient broth.* Details of this are given later.

(2) *Media for the development of L forms from filtrates*

Difco nutrient gelatine was the only medium of the many tried in which development of filter-passing particles was obtained. Various types of plant-tissue media and nutrient broth or agar or synthetic media, with or without the addition of plant-tissue, were all ineffective.

Filters. Fritted glass filters (H 5) were the only satisfactory filters. Filtrates from Seitz filters or Berkefeld filter candles were either almost or entirely free from filter-passing particles, probably owing to their adsorption on the filters.

Cultures. In most of the experiments *C. fascians* strain 538, a virulent strain isolated from sweet-pea galls in 1956, was used. Strain 505 (isolated from sweet-peas, 1949) was used in addition to strain 538 in a penicillin broth experiment. Other strains, details of which are given in Table 1, were tested in the final stages of the work.

Cultural conditions. Cultures were incubated at 24° C. for periods varying from 24 hr. to 15 weeks, then passed through glass filters after clearing through filter-paper. The filtrates were pipetted on to slopes of Difco nutrient gelatine or mixed with the melted gelatine and set before incubation. It was found that the gelatine set firmly if 2 ml. of filtrate was added to 6 ml. of medium.

In the early experiments a number of other media were also inoculated with the filtrate, but as it was found that growth of filter-passing particles only occurred in the gelatine medium this was used exclusively in the later tests except for the inoculation of nutrient agar and dextrose potato agar as checks for normal bacterial growth.

EXPERIMENTAL

The initial experiments were made with cultures grown on (*a*) gall-tissue media, and (*b*) penicillin broth and with a filtrate from a naturally occurring leafy gall (*g*).

(*a*) Good growth of characteristic *C. fascians* type (a surface film of yellow growth with clear fluid below) developed in flasks of water containing sweet-pea galls. After 3–4 weeks' incubation the culture showed many pleomorphic forms—swollen, irregular, beaded rods, cocci and filaments—and masses of granules, either free or still enclosed in a bacterial cell-wall. When this stage was reached the cultures were filtered.

(*b*) *Penicillin broth cultures.* Culturing organisms in media containing sublethal doses of penicillin has been found to induce the L phase in several bacterial species (Dienes & Weinberger, 1951). This method was tried with two *C. fascians* strains (538 and 505), which were grown in nutrient broth containing 0·125 unit of penicillin per ml. of broth. After initial inhibition the cultures grew well. They were filtered after 6 days' incubation, when many pleomorphic forms, similar to the (*a*) cultures, were present.

(*g*) *Gall-extract filtrate.* A large geranium gall, from which a virulent culture of *C. fascians* was isolated, was crushed in water, left for 3 hr., then filtered through a Seitz filter pad.

The filtrates were inoculated on to a number of different media. No growth occurred in any except in cultures of nutrient broth and gelatine to which surface-sterilized germinated sweet-pea seeds were added. In no case was there any normal bacterial

growth, but a very thin film slowly developed round the pea tissue (Pl., fig. 1). This gradually spread over the surface of the gelatine, which became softened, and slight general cloudiness developed in the broth cultures. Subcultures from the films again failed to grow except when surface-sterilized germinated sweet-pea seeds were added to the media. Subcultures were carried on in this way for several generations, the same thin film developing round the pea tissue. Microscopical examination of the early stages of growth showed amorphous, faintly staining material and granules of various sizes but no normal bacteria. Later, coccoid-like elements and an occasional rod-shaped form were seen. Cultures from the penicillin broth and gall-extract filtrate developed the granular growth only, except in one subculture in which a yellow coccus developed round the sweet-pea tissue. At this time, this was thought to be a contamination on the sweet-pea seed. Of five filtrates from gall-tissue cultures, one was completely negative and three produced the granular phase only. Cocci developed in subcultures on gelatine + sweet-pea tissue from the fifth filtrate, and in one of these there was finally complete reversion to typical *C. fascians* rods, which grew normally on standard nutrient agar.

The sequence of growth in these cultures was strongly suggestive of the development of filterable particles of the L phase of bacteria, but as it only occurred in media to which sweet-pea seeds were added the evidence was not conclusive; there was a possibility that the growths derived from organisms inside the seed-coat which had survived sterilization (although seed contaminations which occurred in a few cases were of normal saprophytic species of bacteria and very dissimilar to the thin film of granular particles of the filtrate cultures). This doubt was finally dispelled by Expt. 9, in which the same sequence of growth was obtained in gelatine media without the addition of living plant tissue.

Experiment 9

Sweet-pea galls in sterile tap-water were incubated at 24° C. for 7 weeks. The galls remained firm with no sign of necrosis, but a thick growth of *C. fascians* developed on the surface of the liquid. The fluid was passed through two fritted glass filters.

Inoculation

(1) 2 ml. of each filtrate was inoculated on to a variety of culture media; negative results were obtained in all except the four nutrient gelatine cultures. After 10 days incubation the top 1 ml. of the gelatine became of a treacly consistency and the four cultures were completely liquefied after 6 weeks. At first, the medium was quite clear, but after 12–18 days the liquefied gelatine became slightly opalescent, then distinctly cloudy and finally turbid with a white deposit, which consisted of masses of granules of varying sizes, but no definite bacterial forms. After 10 weeks, Gram-negative motile cocci appeared in all four cultures. The coccal form persisted in all subcultures from two of these, but in the other two regeneration into typical *C. fascians* rods was finally complete.

(2) A week after filtering, four more gelatine tubes were inoculated with filtrate. Liquefaction of the gelatine and the growth of granular particles occurred in all. Three did not develop further but the motile coccus appeared in the fourth culture.

(3) Cultures on gelatine were made 3 and 6 weeks after filtering from filtrate kept in sterile bottles. In both cases the gelatine was liquefied but remained perfectly clear. The filter-passing particles were therefore no longer viable.

(4) 6 weeks after inoculation, all tubes of nutrient agar, potato-dextrose agar and *Rhizobium** medium appeared sterile. Difco gelatine was melted and poured into one culture of each. All cultures to which the gelatine was not added, and the nutrient agar + gelatine culture, remained sterile until discarded 3 months later, but the gelatine in the potato-dextrose agar culture liquefied and became cloudy with granular growth, and one typical *C. fascians* colony developed after 1 month on the surface between the agar and gelatine in the *Rhizobium*-medium culture. Evidently, a few filter-passing particles remained viable, but did not develop, in potato-dextrose agar and rhizobium media for 6 weeks; a considerably longer period than in filtrate alone.

A number of similar experiments were carried out to confirm the results obtained in Expt. 9 and to find the optimum conditions for the development of filter-passing particles in pre-filtrate cultures and for their growth after filtration. The best results were obtained from filtrates of cultures of unsterilized sweet-pea galls in water. Cultures were examined microscopically at frequent intervals for the appearance of cocci and granular masses. In the later tests, cultures were also examined for gelatinase which was found to be necessary for growth; the softening of the gelatine in filtrate cultures invariably preceded, for several days at least, the clouding of the media which denoted the appearance of granular particles. The gradual softening and treacly consistency of gelatine inoculated with filtrates bears a very close resemblance to the effect of *C. fascians* strains on gelatine. This organism does not liquefy gelatine held at 20° C. but after 2–4 weeks' incubation at 24° C. the medium becomes of a thick treacly consistency (Lacey, 1939). It was found that no gelatinase was present in any of the plant-tissue media (sweet-pea galls, *Phaseolus vulgaris* shoots or Brussels sprouts) before inoculation, but was slowly produced as a product of bacterial growth, the time varying with different *C. fascians* strains and in different media. Heating a filtrate in a boiling water-bath for 10 min. destroyed the gelatinase, but subcultures of the granular deposits from filtrate gelatine cultures on to a heated filtrate and gelatine medium were negative; there was no liquefaction and no further growth of the particles. It was thought that substances produced by the partial break-down of gelatine might be essential for the development of the filter-passing organisms. To test this, gelatine that had been liquefied but remained free of growth, and also gelatine cultures just beginning to show signs of growth but heated to destroy living organisms, were inoculated with filtrates from various cultures which had failed to liquefy gelatine and also with granular particles. No growth was obtained in these.

Summary of growths obtained from sixteen culture filtrates

(*a*) Four out of sixteen filtrates from glass filters were completely inactive; there was no softening of the gelatine and no signs of growth in any medium. These four negative filtrates were all from cultures on plant-tissue media which had been sterilized in the autoclave.

* As used by Lacey (1955) for development of acid-fast organisms in *C. fascians* cultures.

(*b*) Gelatine cultures of the remaining twelve filtrates developed exactly the same pattern as in Expt. 9; a gradual softening of the clear gelatine was followed after varying periods of time by a slight cloudiness due to amorphous, faintly staining material. In most cases this increased until there was general turbidity throughout the medium, often with the formation of a deposit consisting of particles of varying sizes.

(*c*) Two of the filtrates did not develop further in any culture. The liquefaction and subsequent clouding of the gelatine by particles persisted in subcultures on gelatine to the 4th transfer but no normal bacterial growth was obtained.

(*d*) Cocci developed in five of the filtrate cultures. These were finally induced to grow, though very feebly, on potato-dextrose agar.

(*e*) After a number of subcultures of the cocci, three of the five developed a few colonies with the characteristic morphology and grouping of *C. fascians* rods. These grew well on nutrient agar, showing complete reversion to the parent type.

(*f*) Typical *C. fascians* developed without the intermediate coccal stage in one culture, when gelatine was added to a filtrate culture on potato-dextrose agar which showed no signs of growth after 6 weeks' incubation. The gelatine softened and became cloudy with particles, then one colony of *C. fascians* developed on the interface between the gelatine and agar. (No normal bacterial growth was obtained from any of the other gelatine cultures of this filtrate.)

(*g*) Thin Gram-negative rods developed after the granular phase in all the gelatine cultures of four filtrates. Unfortunately, all the cultures quickly died out and no further study of these forms was possible.

Strain 538 was used in all the above experiments. In a final test seven other *C. fascians* strains in addition to strain 538 were examined for filter-passing particles. The strains were grown in 150 ml. conical flasks containing 50 ml. of brussels sprouts medium. The cultures were tested at intervals for gelatinase, which was produced earlier and in greater amount in the 538 culture than in the other seven. However,

Table 1. *Comparison of eight* C. fascians *strains for filter-passers*

(−, negative; ±, slight; +, fair; ++ strong.)

				Filtrate cultures	
Strain	Date of isolation	Host plant	Gelatinase development	Granules developed in gelatine media	Regeneration of bacteria
538	1956	Sweet-pea	++	+, all cultures	.
448	1938	Petunia	+	3+, 3−	Cocci 2, *C. fascians* 1
505	1949	Sweet-pea	+	+, all cultures	Cocci, all cultures
541	1957	Geranium	+	+, all cultures	.
531	1953	Calendula	±	3+, 3−	Cocci, 1
22	1957	Dahlia	±	±, all cultures	. *
100	1951	Strawberry	−	.	.
405	1937	Nicotiana glutinosa	−	.	.

* Although no bacterial forms were seen in the gelatine cultures of strain 22, diplococci developed in 5 ml. of filtrate to which a nutrient solution of glucose, bacto-tryptone and yeast extract was added. These did not grow on subculture.

five of these gave a positive reaction after 7–9 weeks' incubation. The cultures were filtered as soon as the reaction was well established. Two strains were still negative after 10 weeks, when they were filtered. The filtrates were inoculated on to Difco gelatine, potato-dextrose agar and nutrient agar immediately after filtering and some further inoculations were made subsequently from filtrates kept in sterile bottles. Table 1 shows the results. All the strains were pathogenic to sweet-pea on isolation; three had subsequently lost all virulence.

The table again shows the connexion between gelatinase and the development of filter-passing particles, strains 100 and 405 being negative in both respects. No correlation is found between virulence or length of time a culture was kept on artificial media and ability to produce filterable elements, for strain 448, isolated in 1938 and now completely non-pathogenic, was second best to 538 in time and amount of gelatinase production and developed typical granular growth in filtrate cultures, with complete regeneration of *C. fascians* type bacilli in one. Strain 405 was negative in every respect as was strain 100, which when isolated in 1951 was very virulent but completely lost pathogenicity in a few months.

MORPHOLOGY, PHYSIOLOGY AND PATHOGENICITY OF CULTURES DEVELOPED FROM FILTER-PASSING PARTICLES

Morphology

The bacteria found in completely reconstituted cultures were identical morphologically with the type *C. fascians*, i.e. non-motile Gram-positive rods of variable length and width, frequently exhibiting polar or central staining or banding, arranged in characteristic V, W or X groups. The motile Gram-negative cocci which were the first organisms to appear in the granular masses of the first growth phase were so dissimilar to the parent culture that their origin was considered doubtful until their frequent occurrence and the fact that they could not be identified with any known coccal species gave such strong support to the supposition that they were a phase in the life-history of *C. fascians* L forms. Many microscopical examinations of coccal cultures grown under various conditions and of different ages were made in the search for transitional forms. In some subcultures of Expt. 9 the coccal stages persisted, and the cocci were still motile, after 2 years' cultivation on artificial media; usually, however, motility was soon lost in subcultures. Occasionally films of cultures showed all stages from cocci to long rods with some of each type staining Gram-negative, Gram-positive or indefinite, the appearance being that of a very mixed bacterial growth (Fig. 8). Some old cultures showed groups of Gram-positive rods with a background of degenerating Gram-negative cocci. Occasionally, typical *C. fascians* cultures were obtained from these but sometimes the organisms were not viable.

Physiology

C. fascians is physiologically inert. Slight acid may be produced in synthetic sugar media but in peptone sugar cultures the reaction remains neutral. An alkaline reaction is the only change apparent in litmus milk, no indol is produced and nitrate is very feebly reduced to nitrite, again in synthetic media only.

The fully reconstituted *C. fascians* cultures grew as well as the parent strains on most media but the reactions of the coccoid phase were difficult to obtain as growth was always feeble even under the most favourable conditions and many cultures failed to grow at all on the various 'identification' media. Finally, five cultures of cocci were obtained which were induced to grow on a peptone yeast-extract medium containing a sugar or potassium nitrate. These five cocci cultures and five fully reconstituted strains were compared with the parent strain 538 on the various media and found to be identical, with the exception of the power to reduce nitrate. Strain 538 (and also strains 505 and 448) and one of the reconstituted cultures agreed with the type *C. fascians* in not reducing nitrate in a peptone medium, but the other four reconstituted cultures and all five of the cocci gave a definite nitrite reaction after 4–11 days' growth. Strains 538, 505 and 448 were then compared with three of the filtrate cultures on a synthetic nitrate medium. Although the filtrate cultures grew very feebly on this medium they all gave a slight positive nitrite reaction on the 4th day. Strains 538, 505 and 448 cultures were negative until the 11th day, when a trace of nitrite was present, decidedly less than in the filtrate cultures. Thus nine out of ten filtrate cultures had developed a definite increase of ability to reduce nitrate to nitrite.

On gelatine, all strains gave the characteristic *C. fascians* reaction—i.e. no true liquefaction but a slow softening of the gelatine to a treacly consistency after 2–4 weeks' incubation at 24° C.

Pathogenicity

The cocci-phase cultures and the fully regenerated forms were all avirulent to sweet-pea seedlings. The parent cultures (538 and 505), tested at the same time, produced typical galls on similar seedlings.

DISCUSSION

The development of growths in *C. fascians* filtrate cultures is identical with that of the L phase described by workers studying animal pathogenic bacteria. Kleinberger-Nobel (1951) says: 'In a gram-stained smear of an L culture only a faint cloudiness can be discovered. Its cytoplasmic elements are amorphous in that they can take any shape and can be of large or very small size.... They can transform directly into bacilli or can... reproduce their own kind directly by segmentation and by the production of minute granules which can grow out again into the cytoplasmic elements.' This might be a description of the first phase of the development of filter-passing particles in gelatine filtrate cultures of *C. fascians*.

As the affinity of *C. fascians* to the acid-fast group of bacteria has been noted in earlier work (Lacey, 1955), the close similarity of the filtrate cultures of *C. fascians* to a granular phase frequently found in this group is of particular interest. Kleinberger-Nobel (1951), in a review of the filterable forms of bacteria, describes the interesting observations made by Kahn (1930) of a growth cycle of the human tubercle bacillus in single-cell culture. Kahn noted the formation of three or four separate ovoid units formed by segmentation of a single tubercle bacillus; these divided further into diplococcal forms, then into tiny cocci or granular forms which clumped into tight masses. Delicate rods sprouted from the periphery of these clumps. These and the granules

were not acid-fast but the rods increased in length and width and produced mature bacilli which were acid-fast. This cycle of growth is exactly similar to that observed in *C. fascians* filtrate cultures. In these cultures the granules, cocci and thin rods were Gram-negative but the fully reconstituted rods were Gram-positive (perhaps comparable to the change in acid-fastness of the tubercle bacillus).

Filtrate cultures generally require subculture through several transfers before normal bacterial cells appear, but Tuckett & Moore (1959) found a culture of *Cellvibrio gilvus* which regularly produced viable filtrable particles from which the normal parent type was regenerated immediately. Dark-ground observation suggested the existence of a complex life-cycle. Barely visible particles enlarged into normal cells, these swelled into large bodies in which particles and cells appeared. These became highly motile and emerged through the flexible wall of the body. The granules ranged in size down to the limits of visibility. They found that of ten fritted glass filters used in this work, two were impenetrable to any *C. gilvus* particles, three allowed passage of particles in every test and five were variable, evidently borderline cases between sizes that admitted the smallest particles and those that passed none. They say 'in case of one of two particles admitted by the filter it would be a matter of chance whether they remained viable long enough to regenerate the bacterial form'. This would explain the frequent failures to obtain comparable results on repetition of a successful experiment experienced by workers investigating the problem of filter-passing bacteria.

In the present work the best results were obtained from filtrates of gall cultures. This suggests that the L phase of *C. fascians* is developed to a large extent in the galls. The presence of filter-passing particles in galls was demonstrated in an early experiment in which the granular phase developed in filtrate cultures from a geranium gall. In this case, growth developed only round germinated sweet-pea seeds embedded in gelatine. Subsequently, it was found that filter-passing particles would grow on gelatine without the addition of living tissue if the filtrates contained gelatinase, which was formed after a period of incubation of the galls in water. Filtrates of sweet-pea galls crushed in water and filtered without incubation did not contain gelatinase and gave negative results. This suggests that the partial break-down of the gelatine results in the formation of some substance necessary for growth of the L phase and that the essential growth-promoting substance was provided by the sweet-pea tissue in the earlier experiments. The fact that gall-extract filtrates may produce typical galls on sweet-peas, although cultures of the filtrates remained sterile, is also evidence for the existence of the L phase in gall tissue. In this connexion some work reviewed by Kleinberger-Nobel (1951) is of interest. Acid-fast bacteria are rarely demonstrated in lung nodules of cattle suffering from Perlsucht or in cold abscesses of humans but non-acid-fast granules composed of very small elements present in the lesions were found to be virulent. Failure to isolate *C. fascians* from typical leafy-galls is not uncommon. If the organisms in the galls are in the L phase of their life-history this failure would be explained, as the granules would not grow on the isolation media.

Another puzzling feature of *C. fascians* galls has been the failure to demonstrate any organisms in the interior of the growths during the period of active development of the galls (Lacey, 1936). The granular phase of *C. fascians* is Gram-negative and would therefore not be distinguished from the normal granules found in actively dividing

meristematic tissue. Also, again, cultures made from the interior of the galls would remain sterile.

Occasionally, typical *C. fascians* on isolation plates from galls are avirulent. It has also been noted that regenerated *C. fascians* strains from filtrate cultures had lost virulence. This loss of virulence has its counterpart in animal pathology for Kawatomari (1958) found that six reverted strains of *Clostridium perfringens* from L forms induced by growth of six types of the organism in penicillin plate culture were completely non-toxic to rabbits and guinea-pigs; the original cultures were all toxic.

The existence of the L phase in the life-history of *C. fascians* and the possibility of its frequent occurrence in galls may help to elucidate some of the many problems involved in the study of the activities of this organism.

Finally, one experiment made with a filtrate from *Agrobacterium tumefaciens* galls in the initial stages of this work should be noted. Galls produced on carrot-slices by inoculation of *A. tumefaciens* were chopped up and extracted in sterile water for 24 hr. The fluid was passed through a glass filter and the filtrate inoculated on to a variety of media, including four tubes of Difco gelatine. The results were identical with those obtained later with *C. fascians* filtrates, i.e. no bacterial growth was obtained in any of the media, but in all the gelatine cultures the gelatine was liquefied and a white deposit slowly developed; this consisted of granules of various sizes. No normal bacterial forms developed and no further growth was obtained in subcultures of the granules. This was the first experiment in which this phenomenon had been observed and the result was incomprehensible, particularly as the parent culture *A. tumefaciens* did not liquefy gelatine. It now appears probable that the L phase is a stage in the life-history of *A. tumefaciens*, and may be found in galls produced by this pathogen as well as in *C. fascians* galls.

I would like to take this opportunity to thank Dr S. E. Jacobs for his interest and advice.

REFERENCES

Dienes, L. & Weinberger, H. J. (1951). The L forms of bacteria. *Bact. Rev.* **15**, 245.

Kahn, M. C. (1930). A growth cycle of the human tubercle bacillus as determined by single-cell studies. *Tubercle*, **11**, 202.

Kawatomari, T. (1958). Studies on the L forms of *C. perfringens*. *J. Bact.* **76**, 227.

Kleinberger-Nobel, E. (1951). Filterable forms of bacteria. *Bact. Rev.* **15**, 77.

Lacey, M. S. (1936). Further studies on a bacterium causing fasciation of sweet peas. *Ann. appl. Biol.* **23**, 743.

Lacey, M. S. (1939). Studies on a bacterium associated with leafy galls. Part 3. Further isolations, inoculation experiments and cultural studies. *Ann. appl. Biol.* **26**, 262.

Lacey, M. S. (1955). The cytology and relationships of *Corynebacterium fascians*. *Trans. Brit. mycol. Soc.* **38**, 49.

Tuckett, J. D. & Moore, W. E. C. (1959). Production of filterable particles by *Cellvibrio gilvus*. *J. Bact.* **77**, 227.

EXPLANATION OF PLATE

(Magnification of figs. 2–8, × 1520.)

Fig. 1. L-phase growth of amorphous material and granules developing round a sweet-pea seed in gelatine.

Fig. 2. Granules in an L-phase culture.

Fig. 3. Groups of cocci in amorphous material.

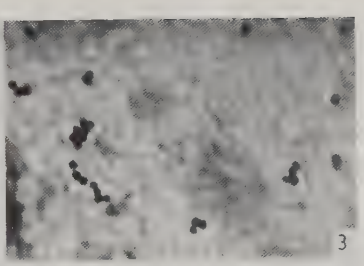
Fig. 4. Cocci; single, in pairs or short chains.

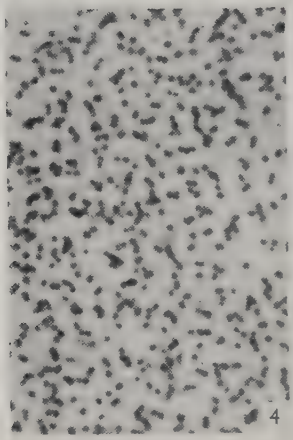
Fig. 5. Coccus with peritrichous flagella.

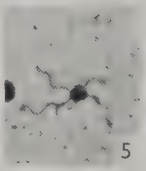
Fig. 6. Rods developing in a coccal culture.

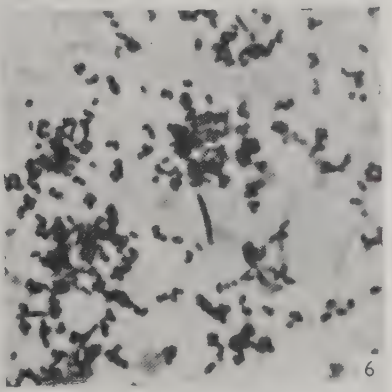
Fig. 7. Short rods showing characteristic *C. fascians* grouping.

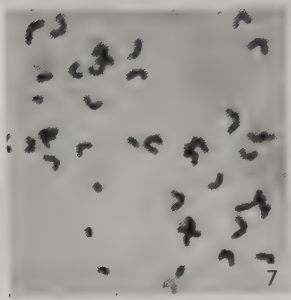
Fig. 8. All stages; cocci to long rods.

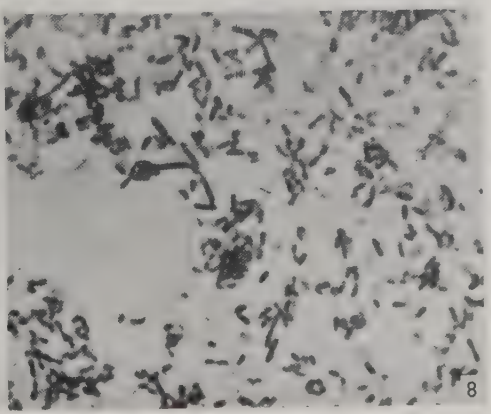












MARGARET S. LACEY

(*Facing p.* 644)

Ann. appl. Biol. (1961), **49**, 645–658
With 1 plate
Printed in Great Britain

Dispersion and deposition of airborne *Lycopodium* and *Ganoderma* spores

By P. H. GREGORY,* T. J. LONGHURST† and T. SREERAMULU‡
Department of Botany, Imperial College of Science and Technology, London, S.W.7

(*Received* 18 *March* 1961)

SUMMARY

Spores were liberated at 0·25 and 1·0 m. above grass. Airborne concentrations at twenty or more points in the spore cloud were measured with specially designed and calibrated miniature suction traps, operated off a portable vacuum system. Deposition in the downwind direction was measured on artificial surfaces on the ground. Measurements were made up to 10 m. from the source.

The observed increase in horizontal cross-wind standard deviation of the *Lycopodium* cloud agreed well with O. G. Sutton's theory, but was incompatible with that of W. Schmidt.

The coefficient of deposition, p, calculated from mean cloud concentration per c.c. and deposition to ground per cm.^{2}, as well as the velocity of deposition, v_g, varied with distance from source. Both p and v_g were larger for *Lycopodium* than for *Ganoderma*, and at the greater distances tested, v_g approximated to the expected terminal velocities of the particles in still air.

The total number (Q_0) of *Lycopodium* spores liberated in each experiment was known, and tentative estimates of the proportion deposited within the sampling area indicated that, under day-time winter conditions in England, the cloud lost not less than 13 to 24% of its load within 10 m. of the source.

INTRODUCTION

When a cloud of spores in suspension in moving air passes over the ground some of the spores are deposited on soil, vegetation and other surfaces. The quantitative relation between the numbers of spores carried in unit volume of air and the number deposited per unit area of surface has scarcely been investigated, although it is of considerable biological interest. A measure of this deposition was derived by Gregory (1945) using results of experiments with *Tilletia caries* spores by Stepanov (1935). Simultaneous measurements of cloud concentration and deposition in the field have been reported by Chamberlain (1956), who used *Lycopodium* spores.

Two measures of deposition have been suggested. The deposition coefficient, p, is the equivalent thickness of the slice of spore cloud cleared in travelling over unit area

* Now at Rothamsted Experimental Station, Harpenden, Herts.
† Now at 31 Bulkington Avenue, Worthing, Sussex.
‡ Now at Department of Botany, Andhra University, Waltair, India.

of ground under a given set of conditions (Gregory, 1945). For convenience centimetre units have been taken and then

$$p = \frac{\text{number of spores deposited per cm.}^2 \text{ of ground}}{\text{number of spores in suspension per cm.}^3 \text{ of cloud}},$$

or, in the terms used by Gregory & Stedman (1953),

$$p = \frac{\text{trap dose}}{\text{area dose}}.$$

Under streamline conditions the coefficient of deposition could be calculated as the resultant of wind-speed and terminal velocity of fall of the spores, but under turbulent conditions outdoors this assumption may not be valid, and experiments are needed to determine the effects of particle size and wind-speed. Data from experiments with *Tilletia* spores by Stepanov (1935) gave a preliminary estimate of $p = 0{\cdot}05$ in centimetre units; this means that in travelling across 1 cm.^{2} of surface the spore cloud would deposit a quantity of spores approximately equivalent to the number contained in a slice 0·5 mm. thick under the experimental conditions (Gregory, 1945).

Another measure, the velocity of deposition, used by Chamberlain (1956), was defined as

$$v_g = \frac{\text{amount deposited per cm.}^2 \text{ of surface per second}}{\text{volumetric concentration per cm.}^3 \text{ above surface}}.$$

In field tests with *Lycopodium* spores marked with iodine-131, Chamberlain measured velocities of deposition ranging from 2·07 to 0·50 cm./sec. at a distance of 20 m. from the point of liberation.

In the experiments described below with spores liberated in the open air, spore concentration and deposition were measured simultaneously at several points downwind of the source, and at the same time information was obtained on the diffusion of spore clouds over the ground for comparison with theories of eddy diffusion.

METHODS

Eleven tests were made with spores of either *Lycopodium clavatum* or *Ganoderma applanatum*, liberated from artificial spore-ejectors at a short distance above ground level and allowed to diffuse into the wind. To estimate spore concentration, samples of air were drawn into miniature suction traps, placed at distances ranging from 2·5 to 10 m. from the source for *Lycopodium* and 1 to 5 m. for *Ganoderma*, and in a number of directions radiating from the source. In addition, on the downwind radius only, spore deposition was measured on artificial sticky surfaces on the ground. These two measurements suffice to estimate p and v_g.

Experimental site

All experiments were done over short, rough grass occupying part of a 4-acre field, surrounded by tall trees, at Imperial College Field Station, Sunninghill, Berks.

Source of spores

In each of Expts. II–VII, 10 g. of a commercial sample of spores of *Lycopodium* were liberated, giving an estimated source strength (Q_0) of $9{\cdot}39 \times 10^8$ spores. The spores, which are roughly tetrahedral with one convex face, have a diameter of approximately 32 μ. Their density is 1·175, and their terminal velocity of fall in still air is given as 1·76 cm./sec. (Zeleny & McKeehan, 1910).

In Expts. VIII–XI undetermined numbers of spores of *Ganoderma applanatum* were liberated. The spores were obtained by washing leaves, twigs and bark, collected from underneath several fruit-bodies of the fungus, in 80% alcohol. The suspension was filtered through glass-wool to remove debris, and roughly standardized by colour. *G. applanatum* spores average $10{\cdot}5 \times 6{\cdot}5\ \mu$, and their thick, dark inner spiny wall makes them easy to distinguish for counting.

Spore liberation

Lycopodium spores were placed on a diaphragm made of filter-paper held between two pieces of copper gauze over the rim of a plastic (polyethylene) conical funnel. Another funnel was inverted on the top of the diaphragm and the two funnels securely sealed with adhesive tape. The stem of the upper funnel was connected by rubber tubing to the spore outlet which was a piece of glass tubing 1 cm. diameter with a right-angle bend. The stem of the lower funnel was connected by rubber tubing to one of the outlets of a two-way gas tap, and this in turn was connected by 120 ft. of plastic hose to an air compressor. When air was forced through the diaphragm a cloud of spores rose in the upper funnel, aggregates fell back on to the diaphragm and single spores were carried out through the exit tube. The rate of emission was controlled by varying the leak in the two-way gas fitting.

The suspension of *Ganoderma* spores was atomized from a standard medicinal inhaler (Rybar Laboratories Ltd.), and, to avoid spore clumps, the concentration was adjusted so that only about one droplet in ten contained a spore. The atomizer was connected to the two-way gas fitting as for the *Lycopodium* ejector.

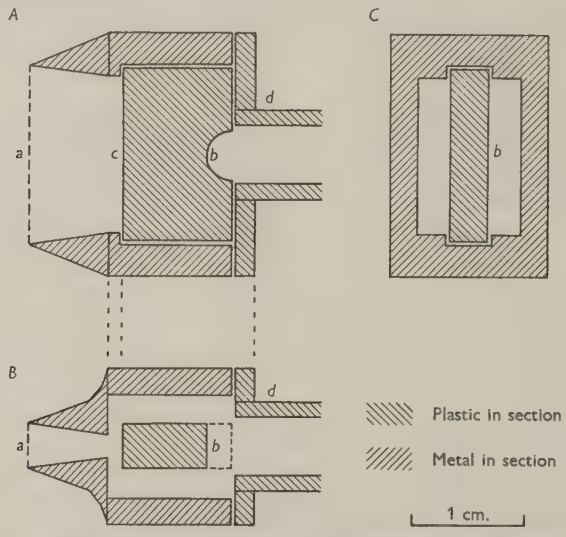
Measurement of concentration in air

To sample a number of regions of the spore cloud simultaneously a suction line apparatus was devised consisting of a number of specially designed miniature suction traps, connected to a vacuum line and run simultaneously off one medium-sized vacuum pump.

The suction line apparatus consisted of the following parts: (1) twenty or more miniature suction traps; (2) an orifice-plate, to stabilize the air-flow rate, connected to each miniature trap by thin-walled rubber tubing; (3) a system of flexible vacuum pipes connected to the orifice-plates; and (4) a single vacuum pump. This system was chosen to avoid the high cost and the individual sampling errors that would be incurred if each trap had a separate vacuum pump.

Miniature suction traps. Each trap consisted of an orifice unit and a back-plate unit (Text-fig. 1 and Plate, fig. A). The orifice unit measured $17 \times 21 \times 15$ mm. externally. The trap-orifice itself was a rectangular opening 16×4 mm., tapering

internally to 14 × 2 mm. A groove on the side walls of the body of the unit extended to within 0·7 mm. of the internal opening of the orifice. A Perspex plate, fitting into the groove, carried a strip of glass coverslip 15 × 3·5 mm. which in turn was fastened (and also made sticky) by dipping in molten petroleum jelly to act as a trapping surface. A semicircle removed from the rear end of the plate allowed unimpeded air-flow to the vacuum line. As it was essential for all the traps to be alike, two brass moulds (one for the orifice and one for the body) were constructed from which any required number of traps could be cast as required in 'Rose's Alloy' (melting point 95° C.), the two pieces being finally cemented together with cellulose adhesive. Each orifice-unit bore a distinguishing number.



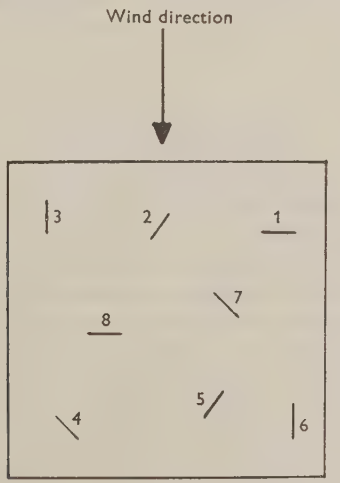
Text-fig. 1. Construction of miniature trap. *A*, Medium horizontal section; *B*, medium vertical section; *C*, section through body. *a*, External orifice; *b*, Perspex plate carrying trapping surface; *c*, adhesive surface; *d*, back-plate unit.

The back-plate (in Perspex) consisted of a rectangular plate, 21 × 15 × 3 mm., with a tube 25 mm. long, 9 mm. internal diameter, cemented into a central hole. In use the back-plate unit was joined to the orifice-unit with adhesive cellulose-tape, care being taken to ensure an air-tight joint.

Orifice-plates. Each miniature trap was connected to the vacuum system through a constricting orifice-plate (not to be confused with the sampling orifice described above) to control the flow rate. These plates were made by drilling 2 in. lengths of aluminium rod with a wide bit, first from one end and then from the other until only a thin partition remained across the centre of the rod. The middle of this partition was then pierced with a hole 0·8 mm. diameter—slightly smaller than required. Each plate was then adjusted individually by connecting one end to the vacuum system and the other to a 'Rotameter'. The diameter of the hole was enlarged with a watch-maker's ream until the flow indicated 10 l./min., the sampling rate chosen for field

experiments. In use each orifice-plate was attached to one end by thin-walled rubber tubing to the back-plate of a miniature trap, and at the other end by pressure tubing to the ultimate ends of the vacuum system.

The vacuum system. For flexibility in the field with negative pressures of up to 12 in. of mercury, the vacuum piping selected was 'rough bore, spiral wire, light-duty suction hose' (Dunlop Rubber Co. Ltd., Manchester; type 427A). The main hose connected to the pump was a 60 ft. length, $1\frac{1}{2}$ in. in internal diameter, the rest of the system consisted of 2·5 and 5 m. lengths with 1 in. internal diameter, the various lengths being connected by galvanized T, Y and X branches fitted with gun-metal connectors (supplied by W. H. Wilcox Co. Ltd., London). After removing a length of the spiral wire, the ends of the hose were forced on to the connectors and secured by worm-drive clips. The ultimate ends of the system were connected to the pressure-tubing leading to the orifice-plates. This vacuum system is adaptable for several experimental designs in the field.



Text-fig. 2. Arrangement of central group of vertical strips on deposition tray.

The vacuum pump. A petrol-driven rotary air-compressor and exhauster, capable of evacuating 500 l./min. (Type NRD, R $3\frac{1}{4} \times 5$, Reavell and Co., Ipswich) was used.

Counting of deposit. After use the miniature traps were taken to the laboratory, the sticky glass slip transferred to a microscope slide, and scanned under the microscope to estimate the number of spores deposited. Knowing the duration for which 10 l./min. had been sampled, and the mean wind speed, it was possible to calculate the mean concentration of the spore cloud as it passed each sampling point.

Measurement of deposition on surfaces

Because of the difficulty of visually scanning the deposit on soil and grass, a simplified model was substituted. A layer of soft modelling wax was placed in a shallow tin tray, $23 \times 22 \times 1{\cdot}8$ cm. On this was placed a sheet of paper marked with a central

square 5 × 5 cm., covered by a sheet of cellulose film ('Cellophane'). In the central area light, thin, wooden or plastic strips, 0·5 × 8 cm., were placed vertically with their bases in the wax as shown in Text-fig. 2 and Plate, fig. B. The strips were blackened to facilitate counting of *Lycopodium* spores by reflected light under the microscope. Fifty similar strips were placed at random around the central square to reduce any edge effect. After exposure to the spore cloud both surfaces of the vertical strips, and also the horizontal cellulose film covering the ground, were scanned microscopically to measure total spore deposition. In Expts. IV–VI with *Lycopodium* spores, deposition was also measured on sticky horizontal microscope slides placed on sheets of glass on the ground: in *Ganoderma* experiments the vertical strips were omitted and only horizontal sticky slides were used.

Measurement of wind-speed

Sheppard cup-anemometers were exposed during each test, at the height of spore liberation and at 1 m., 2 m. and sometimes other heights above ground level to measure mean wind-speed. Wind direction at ground level was determined with smoke from an apiarist's bellows.

CALIBRATION OF MINIATURE TRAPS

As the miniature trap was designed to sample air at 10 l./min., isokinetic sampling was excluded in the field except in winds of 3 m./sec. (the rate of flow into the sampler orifice). The traps were therefore tested against a 'Cascade Impactor' in a small wind-tunnel at various wind-speeds to obtain a calibration curve, enabling efficiency corrections to be applied for each experiment in the field according to the wind-speed.

Tests were done in the wind-tunnel at Rothamsted Experimental Station at four wind-speeds, under controlled conditions of turbulence, using *Lycopodium* spores (for the small spores of *Ganoderma* errors arising from non-isokinetic sampling will be small and were neglected). The miniature trap was operated at its predetermined rate of 10 l./min., while the Cascade Impactor was sampling isokinetically.

The spore deposit from a miniature trap was not uniform; along the 14 mm. length of the spore-trace there were maxima at about 1 mm. from each end, and a tendency for a minimum at the centre of the trace. Sample traces, obtained in winds of 1·9, 3·4 and 6·1 m./sec., were scanned across the trace with traverses 0·2 mm. apart and the total number of spores estimated. It was found that by taking ten equally spaced traverses across the trace and multiplying by the appropriate factor to compensate for the smaller area sampled the estimate was within 2% of that derived from the seventy-one traverses across the same trace: ten counts were therefore adopted in future tests.

Assuming the first two stages of the Cascade Impactor collect and retain *Lycopodium* spores with 100% efficiency, when operated isokinetically, the efficiency of the miniature trap was 54, 74, 85 and 119% at 0·82, 1·95, 3·6 and 6·1 m./sec. respectively. Efficiency increases steadily with wind-speed, and there is evidence of over-sampling (efficiency more than 100%) at speeds above 3·6 m./sec.—an effect possibly exaggerated by the expanded mouth of the trap orifice.

EXPERIMENTS WITH *LYCOPODIUM* SPORES

In conducting an experiment the vacuum-line system was first assembled with branches arranged to allow traps to sample in the required positions. The end of the stem of the system was connected to the suction port of the pump. The pump was started and an air-flow of 10 l./min. through each orifice-plate was verified by connecting each one temporarily to a 'Rotameter' in turn. The pump was then stopped while the miniature traps were fitted in position, and then restarted to draw air simultaneously through all the traps at the same rate. Part of the stream of air from the compression port of the pump was conducted by plastic hose to serve as a compressed-air source for the spore ejector.

Miniature traps were fixed at the required height above ground level by clips attached to bamboo canes. Deposition trays were placed on the ground below miniature traps on the downwind radius only, and care was taken to ensure that they were not shadowed by vacuum hose. When all was ready spore-liberation was started by closing the air-leak in the spore-ejector mechanism.

Expts. I and VII gave information on deposition, but not on the lateral diffusion of the spore cloud. In the remaining experiments with *Lycopodium*, twenty-one miniature traps were operated, arranged at different distances and radii as shown with relevant data in Table 1. An example of the arrangement of traps, with values of area dose and trap dose, is shown for Expt. V in Table 2.

Table 1 summarizes the results of the individual experiments in the form of the standard deviation (σ) of the deposited spores from the mean position at each of the distances, calculated from the usual formula: $\sigma^2 = (x-\bar{x})^2/(n-1)$, hence $(x-\bar{x})$ is the deviation of a deposited spore from the mean position at that sampling distance as measured around the arc. The sum of all these deviations is divided by the total number of spores on that arc. (Either the mean concentration of the spore cloud or the area dose can be used in this calculation.) The crude data can be used in three ways: (1) to compare the observed diffusion with that expected from theories of atmospheric turbulence; (2) to determine the deposition coefficient and velocity of deposition v_g; and (3) to estimate by integration the fraction of the total number of spores liberated which has been deposited within the area covered by the traps.

Comparison of results with eddy diffusion theories

Table 1 gives the calculated standard deviation for each sampling point in Expts. II–VI.

According to the theory of Schmidt (1925): $\sigma^2 = 2At/\rho$, where A = the interchange coefficient of eddy diffusion; t = time; ρ = density of air. Because $t = x/u$ (where u = wind-speed and x = distance), we have:

$$\log \sigma = \tfrac{1}{2} \log x + \tfrac{1}{2} \log 2A/\rho u.$$

If this relation holds true in field tests, plotting experimental data for log σ against log x should give a line of slope $\tan^{-1} \frac{1}{2}$, that is 26° 34′. However, according to the theory of Sutton (1932) $\sigma^2 = \frac{1}{2}C^2x^m$ (where C is a coefficient of diffusion with dimensions $(L)^{\frac{1}{2}}$, and m is a number varying between 1·24 in extremely stable, non-turbulent

Table 1. *Particulars of experiments on dispersal and deposition of* Lycopodium *spores over grass*

Expt. no.	Date	Time start	Duration (min.)	Height of liberation (m.)	Temp. (° F.)	R.H. (%)	Trap height (m.)	Wind speed	C $(m.)^{\frac{1}{2}}$	m	Radii: No.	Radii: Degrees apart	Distance, metres from source		
II	23. xi. 55	14.11	25	0·25	48	64–66	0·25	1·05	0·28	2·12	7	20	2·5	5·0	10·0
													σ 1·20	2·47	5·20
III	24. xi. 55	14.31	16	1·0	46	64–67	1·0	2·64	1·40	1·71	3	45	2·5	5·0	10·0
													σ 2·07	3·28	6·77
IV	17. i. 56	14.42	14	1·0	40	96	1·0	0·40	0·97	1·80	7	20	2·5	5·0	10·0
													σ 1·79	3·39	6·29
V	26. i. 56	14.34	42	0·25	52	80–92	0·25	1·28	0·58	1·99	7	20	2·5	5·0	10·0
													σ 1·76	3·6	6·99
VI	12. vii. 56	13.11	47	1·0	64–66	51–53 Sun	1·0	1·63	0·89	1·66	6	20	2·5	5·0	10·0
													σ 1·09	1·90	3·45

Notes. Expt. I was a preliminary test of uniformity of the miniature traps when connected to the vacuum system in the open air. $Q_0 = 9{\cdot}39 \times 10^8$ in all tests.

wind, and 2·0 under conditions of extreme turbulence. In normal overcast conditions $m = 1{\cdot}75$.) Therefore

$$\log \sigma = \tfrac{1}{2}m \log x + \tfrac{1}{2} \log (\tfrac{1}{2}C^2).$$

If this holds true, plotting observed values of log σ against log x should give a line of slope $\tan^{-1} \frac{1}{2}m$. For values of Sutton's m between 1·75 and 2·0 the line should slope at some angle between 40° 36′ and 45°.

Table 2. *Area dose of* Lycopodium *spores* ($9{\cdot}39 \times 10^8$ *liberated at* 0·25 *m. height*) *in Expt. V, with standard deviation, and trap dose on downwind axis*

	Horizontal distance from source (m.)		
Angle (°)	2·5	5·0	10·0
−80	8,700	3,400	900
−60	12,200	5,200	1,200
−40	14,900	5,300	1,300
−20	15,500	5,800	1,800
[Trap dose	836	158	39]
0	16,700	6,800	1,700
+20	15,600	4,500	1,600
+40	9,900	4,400	1,200
S.D. σ (m.)	1·76	3·60	6·99

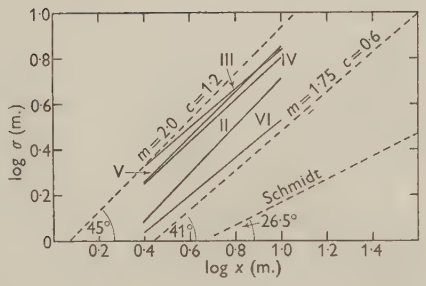


Fig. 3

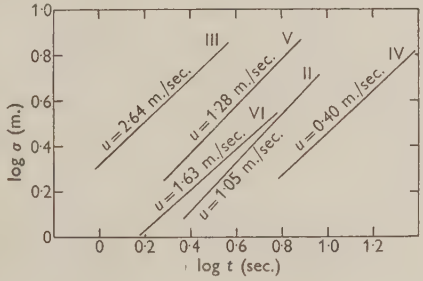


Fig. 4

Text-fig. 3. Plot of log of standard deviation against log of distance in Expts. II–V compared with predictions from theories of W. Schmidt and O. G. Sutton.

Text-fig. 4. Plot of log of standard deviation against log of time showing incompatibility with W. Schmidt's theory.

Experimental values of σ, derived from Table 1, and plotted in Fig. 3, show the lines sloping at angles between 40° and 46°. This is incompatible with the slope of 26° 34′ required by Schmidt's theory, but agrees well with Sutton's theory. Furthermore, according to Schmidt's theory if log σ is plotted against log t (calculated from the distance and mean wind-speed), σ should be the same after a given *time* whatever the wind-speed, but this is not so (Fig. 4). On Sutton's theory at a given *distance* log σ should vary over a comparatively narrow range of values, depending on the parameter m. Evidently under the conditions and limited distance of these experiments, Sutton's theory gives a good prediction of the diffusion of the spore cloud, but the results are incompatible with Schmidt's theory.

Deposition

Some *Lycopodium* spores were deposited on all surfaces exposed, including both sides of the vertical strips, the sticky horizontal slides and the horizontal cellulose film forming the ground layer.

The relative mean deposition of spores on the variously oriented surfaces of the vertical strips is given in Table 3.

Deposition on the front of traps oriented at right angles or at 45° to the wind was several times greater than deposition on the back; but on the two sides of a strip parallel with the wind direction deposition was similar. Between strips in individual experiments catches were very variable, especially at 2·5 m., and more work is needed to explain the reasons for this.

Table 3. *Relative deposition on vertical strips* 8 × 0·5 *cm. in Expts. I–VI*

	Strip orientation relative to wind direction					
	Right angle to wind		Parallel with wind		45° to wind	
Distance (m.)	Front	Back	Left	Right	Front	Back
2·5	100·0	13·2	20·9	25·3	78·0	10·1
5·0	28·6	1·3	46·2	50·6	22·0	2·2
10·0	6·6	1·2	1·0	2·2	6·4	1·0

Table 4. *Deposition on vertical strips as percentage of total for unit area of ground*

Expt. no.	Wind-speed (m./sec.)	(Strip/total) × 100 (%)	p (mean of 3 distances)
IV	0·40	8·4	0·170
I	0·62	8·9	0·068
VI	0·82	14·1	0·041
II	1·09	31·1	0·061
V	1·28	27·5	0·037
III	1·97	27·2	0·016

Deposition to ground and strips compared

Table 4 shows the proportion deposited on strips to the total deposition. The percentage of the total deposition per unit area of ground which is contributed by impaction on the vertical strips increases as the wind-speed increases (an effect to be expected from previous work), though the results suggest that the effect was not measured very accurately.

Calculation of p and v_g

From the area dose and from deposits on trap surfaces, the coefficient of deposition, p, calculated as the ratio of trap dose to area dose, is given in Table 5, where values for velocity towards ground, v_g, are included, as well as mean values of p and v_g for Expts. II–VII at 2·5, 5·0 and 10·0 m. respectively.

Estimation of fraction of Q_0 deposited within sampling area

The sampling area over which the miniature traps were spread is only a small sector of the possible region contaminated by the spore cloud. Using the values of p observed at different distances, it is possible to estimate the approximate number of spores deposited per square centimetre at a point below each miniature trap. Then, by the assumption that the number deposited varies regularly between one trap and the next, it is possible to estimate the total number deposited within the sampling sector and express this as a fraction of Q_0, the total number of spores liberated (Table 6). (In practice this was done by first drawing a graph of the estimated deposit at each sampling point on the arc at each distance from the source, joining the points by straight lines, and summing the area under the curve; then repeating this with the sum for each distance.)

Table 5. *Deposition coefficient, p, for* Lycopodium *spores based on strip+ground deposits; and velocity of deposition, v_g (assuming $v_g = p \times u$)*

	2·5 m.		5·0 m.		10·0 m.		Wind (u) at height of liberation (h)	
Expt. no.	p	v_g	p	v_g	p	v_g	u (cm./sec.)	h (m.)
I*	—	—	0·089	11·6	0·046	6·0	130	1
II	0·13	14·2	0·041	4·5	0·017	1·9	109	0·25
III	0·011	2·9	0·018	4·8	0·017	4·5	264	1
IV	0·440	17·7	0·053	2·1	0·025	1·0	40	1
V	0·054	6·9	0·034	4·3	0·021	2·7	128	0·25
VI	0·015	2·4	0·046	7·5	0·061	10·0	163	1
VII†	0·065	8·8	0·014	1·9	0·061	2·2	135	0·25
Mean	0·119	8·8	0·042	5·2	0·029	4·0	—	—
Mean (II–VII)	0·119	8·8	0·034	4·2	0·026	3·7	—	—

* At 20 m. $p = 0{\cdot}065$, $v_g = 8{\cdot}5$. † At 7·5 m. $p = 0{\cdot}021$, $v_g = 2{\cdot}8$.

Table 6. *Proportion of* Lycopodium *spores deposited within sampling area between 2·5 and 10·0 m. of source*

Expt. no.	Height of liberation (cm.)	Wind-speed (cm./sec.)	Angle of sector (°)	Sutton's C (m.)$^{\frac{1}{2}}$	m	100 × total deposit/Q_0
II	25	109	120	0·28	2·12	24·4
IV	100	40	100	0·97	1·80	18·1
V	25	128	120	0·58	1·99	14·5
VI	100	163	120	0·89	1·66	13·5

Expt. III omitted as only 3 radii were tested.

These estimates, indicating a total deposition within the sampling area varying from 13 to 24% in the different experiments, are merely tentative and to give precision further experiments are needed in which deposition is measured directly (see Sreeramulu & Ramalingam, 1961), and at a greater number of points than in the present series. The values in Table 6 are likely to under- rather than over-estimate the total deposition within 10 m. of the source, because no allowance has been made for deposition closer to the source than 2·5 m., or outside the 100° or 120° sector.

Experiments with *Ganoderma* spores

Experiments with *Ganoderma* spores differed from those with *Lycopodium* in several respects. The *Ganoderma* spores were liberated in minute droplets of alcohol which may be assumed to evaporate after negligible travel downwind. Traps were arranged along one radius only (downwind), no attempt being made to measure lateral diffusion or to estimate c and m of Sutton's equation. Miniature traps and sticky slides were both placed at ground level a few inches apart on a sheet of glass. Attention was thus concentrated on estimating p and v_g from simultaneous measurements of A.D. and T.D. at ground level. In spite of A.D. being estimated below the liberation height, values for p and v_g obtained in the *Ganoderma* experiments were much smaller than for *Lycopodium* (Table 7).

There are at present no comparable data in the literature on deposition of a similar-sized particle. Although results are preliminary, the data show that values of p are approximately one-tenth of those for *Lycopodium*, and follow the same general pattern in decreasing with increasing distance from the source. With deposition expressed as v_g, values ranged from approximately 2·0 to 0·08 cm./sec.—the highest value is therefore ten times the lowest, and the lowest is about half the expected terminal velocity of 0·22 cm./sec. The value of v_g decreases with distance from the source, and only once did the experimental value approach the expected—of the rest eleven were greater and two smaller.

Discussion

Quantitatively the deposition of *Lycopodium* spores varies more than expected. With *Lycopodium* the thickness of the slice cleared, measured by p, varies from 4·4 to 0·11 mm., while the velocity of deposition, v_g, varies from 17·7 to 1·0 cm./sec. Only three of the twenty-two values of v_g are lower than 2·0 cm./sec., which is close to the velocity expected on the gravity deposition theory of 1·75 cm./sec. (the observed terminal velocity of *Lycopodium* spores). At low wind-speeds abnormally high values for deposition tended to occur close to the source, despite the fact that spore concentration was measured on the axis of the cloud and that at these distances from the source the concentration at ground level must be less than on the axis, so the proportion of the cloud at ground level actually deposited must be still higher. This implies that the high values recorded at 2·5 m. are, in fact, under-estimates. So far tests have been done over a small range of wind-speeds only, and it is noticeable that at the higher wind-speeds p and v_g are low at 2·5 m. and higher at 5 and 10 m. At 5 m. or more the minimal values are established and these tend to approach the terminal velocity. The experiments with *Ganoderma* also show greater deposition coefficients close to the source, but there was little variation in wind-speed.

In experiments with *Lycopodium* spores an effect of height of liberation is suggested, deposition rate increasing with increasing distance when liberation was at 1 m. above ground, but when liberation was at 0·25 m. deposition rate always decreased with increasing distance.

The limited data confirm earlier observations that size of spore has little effect on the diffusion pattern or deposition gradient over such short distances as used in these

Table 7. *Experiments on deposition of* Ganoderma applanatum *spores, liberated at height of 0·25 cm., to glass slides on ground*

Expt. no.	Date	Time	Duration (min.)	Temp. (° C.)	R.H. (%)	Mean wind-speed (10 cm.) (cm./sec.)		Distance from source (m.) 1	1·5	2·5	5·0
VIII	10. i. 57	13.40	28	44	64–57	170					
							p	—	—	0·0026	0·0008
							v_g	—	—	0·44	0·14
IX	23. i. 57	12.50	40	34–37	79	62					
							p	0·014	0·010	0·0056	0·0047
							v_g	0·83	0·61	0·34	0·29
X	12. iii. 57	12.42	27	64–65	65–66	91					
							p	0·022	0·0061	0·0051	0·0037
							v_g	1·97	0·56	0·46	0·34
XI	12. iii. 57	13.20	39	64–65	66–65	76					
							p	0·015	0·0056	0·0031	0·0010
							v_g	1·13	0·43	0·24	0·08
							Mean p	0·017	0·0073	0·0041	0·0026
							v_g	1·31	0·53	0·37	0·21

General mean p = 0·0071
v_g = 0·56

tests. Deposition on the downwind axis fell off similarly with both *Lycopodium* and *Ganoderma*, the number of spores deposited per square centimetre at 5·0 m. being 18·4 and 19·8% of the number deposited at 2·5 m. from the source. The diffusion pattern is evidently controlled by atmospheric turbulence, but this does not conflict with other evidence, from measurements of the deposition coefficient, that a much higher proportion of the total number of spores liberated of *Lycopodium* than of *Ganoderma* must have been deposited within the sampling area.

Although *Lycopodium* spores were liberated at 25 cm. or 100 cm. above ground level, the proportion estimated as having been deposited within 10 m. varied from 13 to 24%. Comparable experiments were reported by Stepanov (1935) who made direct counts of deposition on slides placed on the ground over a grass field. Integrating his data for his two experiments (as was done for Table 6) shows that with spores of *Tilletia caries*, liberated at a height of 80–120 cm., 11·2% were deposited within 4·0 m., and 8·6% when liberated at 150 cm. above ground level. More tests are needed with direct measurement of deposition and at a greater range of sampling points to develop this study, which so far supports theories assuming a comparatively rapid loss of spores from the cloud near a ground level source, rather than the very long flight ranges assumed by Schrödter (1960). Obviously many factors enter into the process of spore deposition in the field and we are only at the beginning of the study of this problem.

We wish to thank Mrs Margaret F. Gregory for help with calculations; Mr F. C. Bawden for permission to use the wind-tunnel at Rothamsted Experimental Station; Mr Roy Adams for technical help in laboratory and field; Mr F. G. Smith for making the miniature suction traps; and Mr A. Horne for photography.

REFERENCES

Chamberlain, A. C. (1956). Aspects of travel and deposition of aerosol and vapour clouds. *A.E.R.E. HP/R*, no. 1261, pp. 1–35.

Gregory, P. H. (1945). The dispersion of air-borne spores. *Trans. Brit. mycol. Soc.* **28**, 26.

Gregory, P. H. & Stedman, O. J. (1953). Deposition of air-borne *Lycopodium* spores on plane surfaces. *Ann. appl. Biol.* **40**, 651.

Schmidt, W. (1925). Der Massenaustausch in freier Luft und verwandte Erscheinungen. *Probl. kosm. Phys.* **7**, 1.

Schrödter, H. (1960). Dispersal by air and water—The flight and landing. In *Plant pathology: an advanced treatise*, vol. III, pp. 169–227. Ed. by J. G. Horsfall & A. E. Dimond. New York: Academic Press.

Sreeramulu, T. & Ramalingam, A. (1961). Experiments on the dispersion of *Lycopodium* and *Podaxis* spores in the air. *Ann. appl. Biol.* **49**, 659.

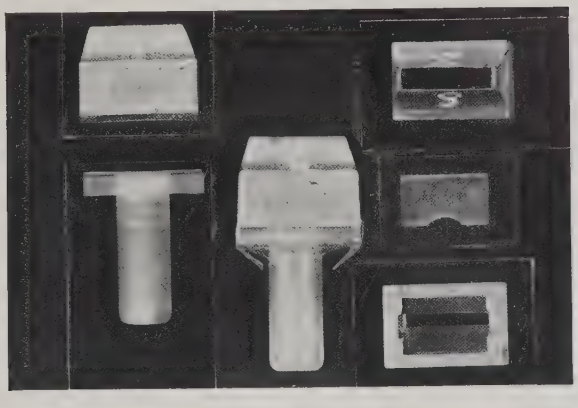
Stepanov, K. M. (1935). Dissemination of infective diseases of plants by air-currents. *Bull. Pl. Prot. Leningr.* Ser. 2, *Phytopathology*, no. 8, pp. 1–68. (Russian, English title.)

Sutton, O. G. (1932). A theory of eddy diffusion in the atmosphere. *Proc. Roy. Soc.* A, **135**, 143.

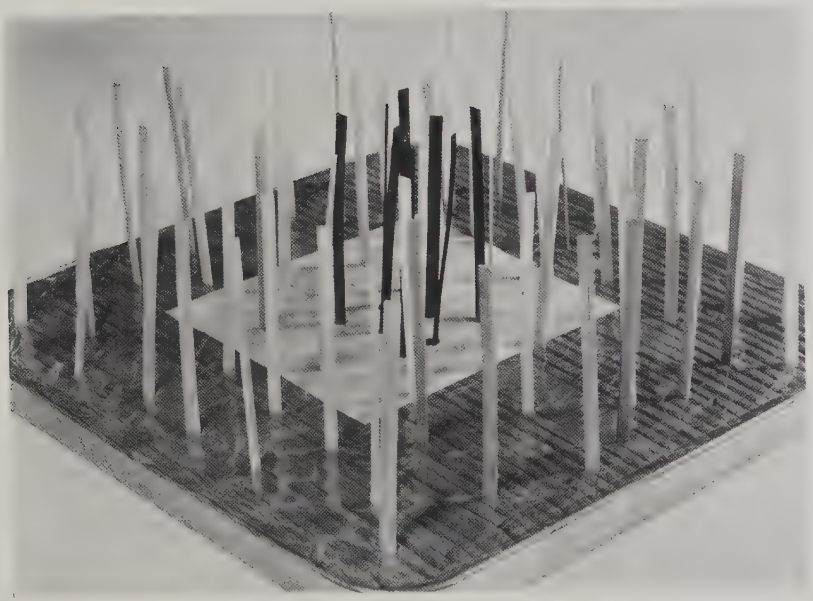
Zeleny, J. & McKeehan, L. W. (1910). Die Endgeschwindigkeit des Falles kleiner Kugeln in Luft. *Phys. Z.* **11**, 78.

EXPLANATION OF PLATE

A. Construction of miniature trap.
B. Arrangement of vertical strips in sampling tray.



A



B

P. H. GREGORY, T. J. LONGHURST AND T. SREERAMULU

(*Facing p.* 658)

Ann. appl. Biol. (1961), **49**, 659–670
Printed in Great Britain

Experiments on the dispersion of *Lycopodium* and *Podaxis* spores in the air

By T. SREERAMULU and A. RAMALINGAM
Botany Department, Andhra University, Waltair, India

(*Received* 18 *March* 1961)

SUMMARY

In twelve experiments known quantities of *Lycopodium* spores (in five with spores of *Podaxis*) were liberated into the open air artificially from a point source, under different meteorological conditions, and trapped on 'Vaseline'-coated slides, placed horizontally at: 2·5, 5·0, 10·0, 15·0, 20·0, 25·0 and 30·0 m. from the source, along from five to thirteen radii (20° apart in some experiments, and 5° or 10° apart in others).

The size of spore had little effect on the relative numbers of *Lycopodium* and *Podaxis* spores deposited at different distances (the deposition gradient), but from a cloud containing ten times as many *Podaxis* as *Lycopodium* spores over twice as many *Lycopodium* were deposited per unit area as were *Podaxis* spores. The rate of deposition is thus approximately proportional to the volume of the individual spore.

Estimated percentage recovery, within the sampling area, of *Lycopodium* spores liberated varied from 5·4% (afternoon in wind of 4·4 m./sec.) to 91·6% (night in wind of 0·8 m./sec.). Recovery of *Podaxis* varied from 0·3% by day to 1·6% by night.

The results of these experiments are examined in the light of the statistical theories of W. Schmidt and O. G. Sutton, which formulate atmospheric diffusion by eddies, and are found to be in closer agreement with the latter. The values of the parameters '*m*' in Sutton's equation agree excellently with his theoretical values, but the values of the parameter '*C*' are more variable.

INTRODUCTION

How far and in what quantity spores can be carried by the wind currents from a particular source is a fundamental question in plant pathology. Determining the limits of protective spraying, delimiting quarantine zones and eradicating intermediate hosts, are all closely bound up with the answer to this problem. Several attempts have been made to formulate laws of dispersal of spores in the air by which the size of the danger zone around a locus of infection could be defined with reasonable accuracy (Stepanov, 1935; Wilson & Baker, 1946; van der Plank, 1948, 1949; Wolfenbarger, 1946; Gregory, 1945, 1952, 1961). Gregory concluded that the laws of diffusion developed in meteorological work could be applied to spore clouds, and for predicting the rate of dilution of the spore cloud as it travels downwind for short distances near the ground adaptations of equations by Sutton (1932, 1947*b*) showed reasonably good agreement with observed concentrations reported in the limited data available from the literature. To obtain a more satisfactory set of data to test the fit of Sutton's theory,

Gregory, Longhurst & Sreeramulu (1961) used suction traps to sample air at different points in a diffusing spore cloud which had been created artificially by liberating spores from a point source under standard meteorological conditions. There are no data yet for conditions when turbulence is at a maximum (as on hot summer afternoons) or at a minimum (as on cold winter nights). In the present work an attempt has been made to collect data to test the fit of the equations under Indian weather conditions.

MATERIAL AND METHODS

Materials

Stakman (1942) noted that many fungus spores do not lend themselves well to investigations designed to elucidate principles for understanding the dispersal process, and stressed the need for 'markers' (spores or pollen grains) that can be identified easily and certainly as of known origin. Spores of *Lycopodium* have been found satisfactory by previous workers because they are readily carried by wind currents and travel separately from each other without clumping. They can be identified easily, and can be purchased in quantity; they number $9{\cdot}39 \times 10^7$ per g. In all the following experiments spores from one commercial sample of *Lycopodium* (B.D.H.) were used. Spores of *Podaxis pistillaris* were also used in some of the experiments for comparing the behaviour of different kinds of spore during their diffusion in air. Air-dried spores of *Podaxis* (which were collected from fruit-bodies growing in Andhra University campus during August–September 1957) after passing through a fine sieve did not show much clumping when liberated and were satisfactory for experimental purposes. A sample, wetted in alcohol, warmed in 10% sodium hydroxide solution and suspended in 0·25% agar water and counted on a haemocytometer slide showed $1{\cdot}8 \times 10^9$ per g.

As the spores had to be liberated uniformly from a point source, a 'spore-ejector', based on the 'boiling-bed' principle, and described by Gregory *et al.* (1961), was used.

Good quality glass microscope slides, 25×75 mm., smeared with 'Vaseline', were used for trapping the spores deposited at each point. The sites selected for these experiments were the hockey and football court on Andhra University campus, about 50 ft. above sea-level and within half a mile of the sea.

Experimental arrangement

The traps used by Stepanov (1935) were placed (presumably horizontally) on the ground at various distances from the source, in various directions relative to the prevailing wind, along an arc at each distance (so the distances from the source were the same on all radii). Wilson & Baker gave a picture of the impaction on vertical surfaces of air-borne spores in the diffusion cone at different distances from the source. Their traps were placed on a line at right angles to the axis of the cone (so that the distance from the source was not the same on all radii). Stepanov's design was chosen in this work because the standard deviation, σ, of the spores from their mean position can be calculated more accurately.

Experimental procedure

Just before starting an experiment the mean direction of the wind was determined with a wind-vane. This direction was taken as the zero radius, identical with the x-axis (the y-axis being horizontally at right angles to this and the z-axis vertical). Using a measuring tape, the trapping points on the different radii were located, and the slides placed in position on small pieces of thin paper-board to prevent dust particles getting on the slides, and also to give a flat, uniform surface for exposure. The labelled, sticky slides were placed in a horizontal position with the label-end pointing towards the same side at all sampling points.

A weighed quantity of spores was placed carefully on the upper side of the paper diaphragm in the spore-ejector, and the bent glass tube, connected to the end of the upper funnel, was directed towards the mean direction of the wind (i.e. towards the 0° axis of the trapping slides on the ground). The height of the spore-liberation point was adjusted to 50 cm., which was the height of the anemometer used to measure the wind-speed during the experiment. The lower end of the spore-ejector was connected with a long rubber tube to the flow-regulating unit which was kept away from the experimental site. The spout of the filter-flask was connected to the compressor-end of the pump. All the connexions were made air-tight so that there was no leak in the system except at the point controlled by the screw of the pinch-cock.

In conducting an experiment the pump was started first and after running a minute or two the time was noted, and spore liberation started by slowly tightening the screw pinch-cock of the spore-ejector unit. The rate of spore-liberation was maintained at the desired level by adjusting the screw. After making sure that all spores inside the spore-ejector had been liberated, the time was again noted and the pump stopped. The slides were then carefully removed from their respective places and were kept in a horizontal position in a dust-proof slide carrier for transport to the laboratory.

The reading of the anemometer, which was installed near the trapping site, was noted at the beginning and end of each experiment, and the mean wind-speed at 50 cm. during the time of the experiment was derived from these values. From wet- and dry-bulb thermometer readings the temperature and relative humidity were measured.

Counting and calculation

To get a reliable estimate of the number of spores deposited per unit area the trap slide was scanned under the microscope using an eye-piece graticule, twenty-eight traverses of the slide being taken at 2 mm. apart along the long axis of the slide. The total area scanned under twenty-eight traverses was 147 mm.^{2}.

As these experiments were arranged like those of Stepanov (1935) the methods for calculating the standard deviation of the deposit and the derivation of the parameters C and m in Sutton's formula: $\sigma^2 = \frac{1}{2}C^2 x^m$, as given by Gregory (1945, pp. 42–8) have been followed. For each experiment a regression line has been drawn to show the relationship between log x and log σ, and the values of the parameters C and m have been derived from these lines.

Table 1. *Particulars of experiments on dispersion of* Lycopodium *and* Podaxis *spores at Waltair, India*

Expt. no.	Date	Time of start (I.S.T.)	Duration (min.)	Design of expt. Angles (°)	Design of expt. No. of radii	Max. distance (m.)	Mean wind-speed (m./sec.)	Temp. (°C.)	R.H. (%)	Weather
II	1. xi. 57	14.10	5	20	8	30	2·1	30	70	Dull previously, bright after 14.00
III	17. xi. 57	15.00	13	20	7	30	2·2	29·9	66	Clear sky, sunshine
IV	18. xi. 57	03.20	12	20	7	30	0·8	25·5	74	Cold night
V	15. xii. 57	12.00	5	20	7	30	3·2	29·0	70	Clear sky, sunshine
VI	17. xii. 57	04.25	25	10	7	45	0·8	24·0	75	Cold night, copious dew
VII	5. ii. 58	12.16	24	20	5	35	3·2	29·0	58	Clear sky, bright sunshine
VIII	6. ii. 58	06.35	30	10	8	35	1·1	21·4–25·0	86–75	Dawn, sun rising
IX	20. ii. 58	15.18	17	10	9	30	4·3	31·0	60	Clear sky, bright sunshine
X	27. iv. 58	13.50	20	10	9	30	3·9	33·5	67	Clear sky, very bright sunshine
XI	3. v. 58	14.24	16	10	9	30	4·7	33·0	67	Clear sky, bright sunshine
XII	23. v. 58	05.00	18	5	13	30	1·8	28·4	82	Clear sky, before dawn
XIII	23. v. 58	14.45	18	10	9	30	4·4	32·5	—	Clear sky, very bright sunshine

Spore source: *Lycopodium*, 10 g. in all experiments. *Podaxis*, 5 g. in Expts. VII–XI only. } In Expts. X and XI not all spores were liberated so Q_0 is unknown.
Height of liberation: 0·5 m. above ground in all experiments.
Site: University hockey court (except Expts. VII–X, and XIII, on football court).

EXPERIMENTAL RESULTS

In these studies, where the design of the experiment and height of spore liberation have been kept constant, data have been obtained under a wide range of conditions, duration of experiment, time of day and speed of wind being the chief variants. For the first time data have been gathered for conditions of low turbulence by doing some tests before dawn.

Table 1 summarizes the relevant conditions for each of the 12 experiments, nos. II–XIII (Expt. I was a preliminary trial to test the method). Data for a typical experiment in which both *Lycopodium* and *Podaxis* spores were liberated simultaneously are given in full in Table 2. The results of all twelve experiments (total catches and standard deviations at each distance) are summarized in Table 3. Standard deviations are given in metres, and the values of the parameter C of Sutton's equation are given in units of: (metres)$^{\frac{1}{2}}$.

Table 2. *Results of Experiment IX*

Angle to wind (°)	Distance (in metres) from the source					
	5	10	15	20	25	30
(*a*) Number of *Lycopodium* spores (Q_0 = 10 g.) deposited on 1·47 cm.^{2} at each point						
−30	9	13	2	0	0	0
−20	12	30	10	4	5	1
−10	46	48	47	30	12	9
0	91	53	53	36	21	7
+10	56	81	33	33	27	7
+20	21	78	31	23	12	6
+30	66	34	20	12	11	3
+40	28	18	4	2	2	0
+50	10	1	0	0	0	0
Total	339	356	200	140	90	33
S.D. (m.)	1·90	3·33	4·16	5·20	7·10	7·68
(*b*) Number of *Podaxis* spores (Q_0 = 5 g.) deposited on 1·47 cm.^{2} at each point						
−30	3	5	0	0	0	0
−20	4	8	9	4	2	0
−10	11	13	31	8	4	3
0	57	20	18	7	5	6
+10	19	18	11	9	8	5
+20	13	28	11	19	2	2
+30	21	11	10	3	4	2
+40	12	5	2	1	1	0
+50	4	1	0	0	0	0
Total	144	109	92	51	26	18
S.D. (m.)	1·79	3·44	4·26	6·03	7·66	7·20

Effect of size of spore on deposition gradient

That the size of spore and velocity of its fall do not affect the spore deposition gradient over short distances from the source has been shown by Gregory (1945) from the data of Stepanov (1935). Comparisons of the percentages of the total numbers trapped at various distances from the point of liberation of a mixed cloud of *Lycopodium* and *Podaxis* spores show that the relative density of the deposit of the two types of spores decreased with increasing distance from the source at almost identical

Table 3. *Total numbers of* Lycopodium *and* Podaxis *spores trapped, and estimated standard deviation at each distance*

Distance from source (m.)	...	2·5	5	10	15	20	25	30	35	40	45
Expt. no.	Spores										
II	*Lyc.* Sum	207	705	316	117	53	23	22	—	—	—
	S.D.	0·77	1·32	2·88	4·36	6·29	7·06	8·93			
III	*Lyc.* Sum	760	658	295	85	58	32	11	—	—	—
	S.D.	1·16	2·74	4·05	5·48	7·34	9·26	14·81			
IV	*Lyc.* Sum	1124	2120	975	461	236	158	143	—	—	—
	S.D.	0·55	0·68	1·22	2·01	2·30	2·30	2·31			
V	*Lyc.* Sum	1140	784	298	71	16	11	6	—	—	—
	S.D.	0·91	1·61	2·94	4·61	5·52	6·45	6·86			
VI	*Lyc.* Sum	545	1812	3008	1946	1218	739	494	310	204	115
	S.D.	0·48	0·73	1·20	1·68	2·34	2·91	3·24	3·88	4·96	4·91
VII	*Lyc.* Sum	123	351	236	117	85	42	31	13	—	—
	S.D.	0·78	1·42	2·44	3·93	5·03	6·87	7·29	7·58		
	Pod. Sum	53	162	92	48	28	16	12	7	—	—
	S.D.	0·73	1·21	2·57	4·07	5·90	5·35	6·76	6·53		
VIII	*Lyc.* Sum	1977	2820	1220	578	296	203	130	69	—	—
	S.D.	0·57	0·89	1·72	2·47	3·35	4·22	4·75	5·65		
	Pod. Sum	995	1413	547	285	184	109	70	53	—	—
	S.D.	0·59	1·28	1·82	2·56	3·37	4·12	4·69	6·34		
IX	*Lyc.* Sum	—	339	356	200	140	90	33	—	—	—
	S.D.		1·90	3·33	4·16	5·20	7·10	7·68			
	Pod. Sum	—	144	109	92	51	26	18	—		
	S.D.		1·79	3·44	4·26	6·03	7·66	7·20	—		
X	*Lyc.* Sum	238	596	298	159	63	39	26	—	—	—
	S.D.	0·95	1·46	2·52	3·39	4·06	5·09	5·44			
	Pod. Sum	87	162	96	49	24	18	11	—	—	—
	S.D.	0·98	1·44	2·64	3·69	4·39	4·11	4·74			
XI	*Lyc.* Sum	177	534	286	157	112	61	35	—	—	—
	S.D.	0·77	1·47	2·12	3·34	4·72	5·94	6·80			
	Pod. Sum	55	191	127	70	39	21	16	—	—	—
	S.D.	0·79	1·56	2·39	3·44	5·27	5·71	5·24			
XII	*Lyc.* Sum	700	1550	987	376	220	120	98	—	—	—
	S.D.	0·81	1·41	2·87	4·70	5·98	7·52	8·52			
XIII	*Lyc.* Sum	201	339	239	123	65	48	31	—	—	—
	S.D.	0·93	1·36	2·59	3·46	4·02	4·18	5·15			

rates, although the volume of a *Lycopodium* spore ($32{\cdot}2\,\mu$) is nearly twenty times that of *Podaxis* ($14 \times 11\,\mu$). Therefore it is concluded that the size of the spore (and the velocity of its fall) has very little effect on the rate of dilution of the spore cloud by eddies over the distances tested. (The rate of loss of spores from the cloud may, nevertheless, depend on spore size as indicated below.) The results of these five experiments, conducted at different times of the day and at different wind velocities, also indicate that the lateral spread of the two types of spores also followed the same pattern.

Table 4. *Values of the parameters C and m in Sutton's equations, and percentage of spores recovered within the sampling area for each experiment*

Expt. no.	Time of start (I.S.T.)	Mean wind-speed (m./sec.)	Spore type liberated	Parameters of Sutton's equations		Percentage of Q_0 deposited within sampling area	
				C (m.)$^{\frac{1}{2}}$	m	*Lycopodium*	*Podaxis*
II	14.10	2·1	*Lycopodium*	0·41	2·01	11·4	—
III	15.00	2·2	*Lycopodium*	0·74	1·83	12·1	—
IV	03.20	0·8	*Lycopodium*	0·39	1·33	45·5	—
V	12.00	2·3	*Lycopodium*	0·60	1·69	10·4	—
VI	04.25	0·8	*Lycopodium*	0·28	1·68	91·6	—
VII	12.16	3·2	*Lycopodium*	0·47	1·81	11·7	—
			Podaxis	0·45	1·80	—	0·48
VIII	06.35	1·1	*Lycopodium*	0·33	1·78	30·0	
			Podaxis	0·41	1·67		1·6
IX	15.18	4·3	*Lycopodium*	0·76	1·56	8·1	
			Podaxis	0·70	1·64		0·3
X	13.50	3·9	*Lycopodium*	0·68	1·44	>6·5	
			Podaxis	0·76	1·33		>0·23
XI	14.25	1·8	*Lycopodium*	0·48	1·75	>7·3	
			Podaxis	0·56	1·62		>0·3
XII	05.00	1·8	*Lycopodium*	0·45	1·96	9·5	—
XIII	14.45	4·4	*Lycopodium*	0·68	1·40	5·4	—

Effect of size of spore on rate of deposition

In the absence of simultaneous measurements of cloud-concentration and deposition on the ground it is not possible to estimate the coefficient of deposition, p (Gregory, 1945). However the relative deposition rates for *Lycopodium* and *Podaxis* can be obtained from the data.

In Expts. VII–XI, 10 g. of *Lycopodium* spores ($32{\cdot}2\,\mu$) and 5 g. of *Podaxis* spores ($14 \times 11\,\mu$) were liberated simultaneously. As the volume of a *Lycopodium* spore is approximately twenty times that of a *Podaxis* spore, it can be estimated that approximately ten times as many *Podaxis* as *Lycopodium* spores were liberated during each experiment. (*Lycopodium*: $9{\cdot}39 \times 10^8$; *Podaxis*: 9×10^9). The experimental data therefore allow a direct comparison between the deposition rates of the two types of spore, and Tables 2 and 3 show that only about half as many *Podaxis* spores as *Lycopodium* spores were deposited per unit area. In the five experiments where both types were liberated simultaneously the total count on the traps was 12,230 *Lycopodium* spores from $9{\cdot}39 \times 10^8$ liberated to 5480 *Podaxis* spores from approximately 9×10^9 liberated.

In other words the rate of deposition of the two types from suspension in the spore cloud was directly proportional to their individual spore volumes, and so also presumably to their terminal velocity of fall in still air.

Cross-wind catches

From experiments conducted at different times of the day it appears that the lateral spread extended to 70° on either side of the mean wind direction at about noon, but the spread was only up to 60° and 50° at 14.00 and 15.00 hr. respectively. During the

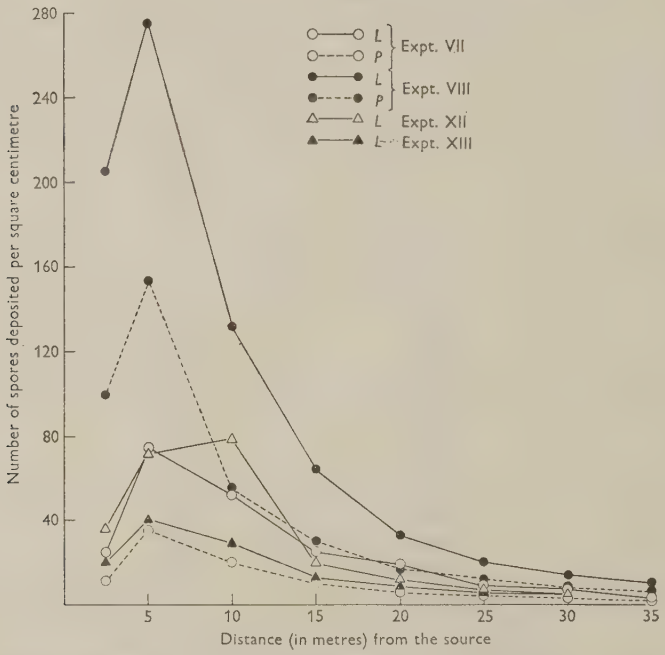


Fig. 1. Effect of size of spore and time of day on downwind catch.

early hours of the morning the lateral spread extended to only 30° on either side of the axis. Lateral spread at the nearer distances, up to 10 m. from the source, was influenced to a certain extent by the mean wind-speed. This was particularly noticeable at 2·5 and 5·0 m. In Expts. II and III, with low wind-speeds (2·1 and 2·2 m./sec.) the lateral spread at 2·5 and 5·0 m. distances extended up to 60°, whereas in Expts. VII and XIII with high wind-speeds (3·2 and 4·4 m./sec.) the lateral spread at these distances extended up to 40° on either side of the mean axis. From 10·0 m. onwards, however, the lateral spread was not much affected by the wind-speed. Duration of spore-liberation also influenced the deposition pattern. When the spores were liberated at a fast rate, as in Expts. II and V (5 min.) the cross-wind catches at nearer distances were large, showing a rapid decrease with increase in distance, but when they were liberated slowly the number deposited was comparatively small and showed a gradual decrease.

Downwind catches

Fig. 1 shows how the downwind catches are affected by the size of spore and the time of day, based on the results of four experiments (Expt. VII at about noon, and Expt. VIII during the following dawn; Expt. XII before dawn, and Expt. XIII during the afternoon of the same day), the total catches on the three radii (20°, 0° and −20°), being common to all these experiments. It is clear from Fig. 1 that as the distance from the source increases the number deposited at first also increases, showing a maximum at 5·0 m., followed by a rapid fall between 5·0 and 15·0 m., resulting in a 'hollow curve' in all the six sets of data. This type of 'hollow curve' is known to depict the general pattern of spore dispersal. The existence of a maximum between 2·5 and 10·0 m. is to be expected from a source elevated at 0·5 m. above ground level (Chamberlain, 1956). In this comparison the size of the spore has no effect in determining the shape of the curve, but the time of day influences both the height and the slope, indicating that before dawn, when the turbulence is very small, many more spores are deposited at nearer distances (within 15 m.) from the source, than during the daytime when the turbulence is known to be high (uniform quantities of 10 g. of *Lycopodium* (*L*) and 5 g. of *Podaxis* spores (*P*) being liberated in all these experiments).

Percentage of spores liberated recovered within the sampling area

In each experiment fifty or so traps were spread out over a sector based on an angle of from 60° to 120°, and extending from 2·5 to 40 m. from the source. Knowing the total number of spores liberated, it is possible to estimate the fraction which had been deposited within the sampling area by applying the method of Gregory, Longhurst & Sreeramulu (1961) directly to the measured deposit at each sampling point. (For Expts. X and XI the values so obtained must be under-estimates, as not all the material was dispersed from the spore-ejector.) The percentage of *Lycopodium* recovered varied from 5·4 to 12·1 % in daytime experiments. At night the proportion tended to be higher (30·0–91·6 %), but one test just before dawn after a warm night gave only 9·5 % recovery. Other evidence during this test indicated a high degree of turbulence ($m = 1{\cdot}96$), whereas the high night recovery values were associated with moderate or low values of m. The recovery of *Podaxis* spores was about one-twentieth that of *Lycopodium*, varying from 0·3 % by day to 1·6 % at night (Table 4).

Comparison with some eddy-diffusion theories

Two divergent theories which formulate atmospheric diffusion by eddies are those of Schmidt and Sutton. Schmidt (1918, 1925) assumes a diffusion constant throughout the process, leading to the expression: $\sigma^2 = 2A/\rho t$ (σ^2 thus increasing as the time, t). Sutton assumed variable parameters of the diffusion equation and concluded that: $\sigma^2 = \frac{1}{2}C^2 x^m$ (σ^2 thus increasing as x^m). The data from our twelve experiments are used to test the fit of these equations.

Schmidt's theory. According to Schmidt's theory plotting the observed values of $\log \sigma$ against $\log t$ should give a straight line of slope 1/2. When this was done (Fig. 2) even though the values in each experiment showed a linear relationship the slope is not 1/2 as predicted by the theory.

Sutton's theory. From the cross-wind data the relation between log σ and log x observed in all the experiments is shown in Fig. 3. To see whether the experimental and theoretical values correspond, lines are also drawn in Fig. 3 for the expected values: (1) for conditions of normal turbulence where $m = 1{\cdot}75$ and $C = 0{\cdot}60\ (\text{m.})^{\frac{1}{8}}$; (2) for conditions of high turbulence with $m = 2{\cdot}00$ and $C = 1{\cdot}10\ (\text{m.})^{\frac{1}{8}}$; and (3) for low turbulence with $m = 1{\cdot}24$ and $C = 0{\cdot}40\ (\text{m.})^{\frac{1}{8}}$. The slopes of these lines for different experiments (indicating the value of m in the equation) are in very good

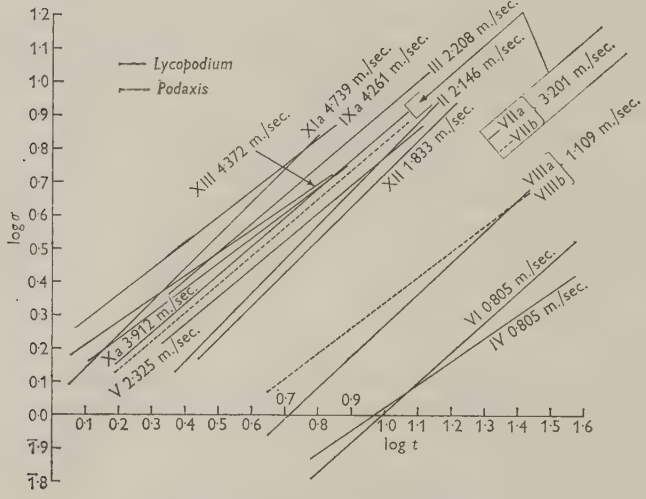


Fig. 2. Observed values of log σ plotted against log t.

agreement with the expected values under different conditions of turbulence and agree well with those obtained from Stepanov's data collected in Russia, and those of Gregory, Longhurst & Sreeramulu in England. The observed values of C, however, show a great variation and more experimental data are required to discover the reasons for this. The small range of values for m, the existence of a continuous variation in the values for C, and the reasonably good agreement in the twelve experiments in India with similar experiments in Russia and England are taken as evidence that Sutton's theory holds good for spore transport in air over short distances of travel near ground level.

Sutton (1932) originally described the parameter C as the general coefficient of diffusion in all directions. Later (1947*a*, *b*, 1953, 1955) he broke C into separate components along the various axes, x, y and z. This is no doubt justified as there is evidence (Scrase, 1930; Wilson & Baker, 1946; Waggoner, 1952) that in vertical diffusion C_z is less rapid than cross-wind diffusion, C_y. For the future a simultaneous study of the horizontal and vertical diffusion should be made so that C can be partitioned into C_y and C_z to test the appropriateness of Sutton's recent treatment for spore transport in air.

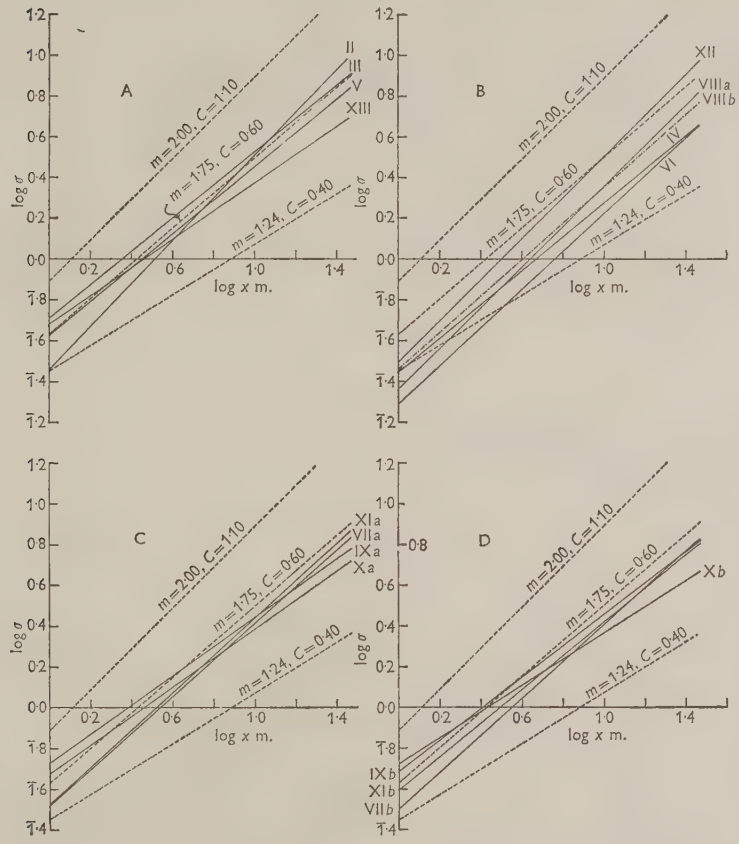


Fig. 3. Relation between log σ and log x, compared with expected values.

DISCUSSION

Although it is not possible to explain the phenomena of dispersal of spores by air currents from the results of these few experiments, the observed deposition gradients indicate that the process to a great extent is described by the theory developed by Gregory (1945), according to which eddies are regarded as the major factor controlling the dispersion pattern. The equations given by him for predicting the number of airborne spores at any distance from a known source are based on different parameters: Q, m, C and p. The recent work of Waggoner (1952) on potato blight indicates that in addition to these another parameter Q_0/k (where k is the ratio of spores deposited per square centimetre to the proportion of leaflets diseased, and Q_0 is the number released at the source) has also to be taken into consideration while dealing with actual disease development in the field. Including this parameter in the Gregory formula, Waggoner derived a modified formula for estimating the number of leaflets becoming

diseased at different distances from the source. The importance of the method of assessment of the disease, and the time at which these assessments are made, in altering the form of the disease gradient under field conditions, has been pointed out by Cammack (1958). If values of these parameters in the above equations are derived by careful assessment of the disease in the field, in relation to the existing conditions of the atmosphere, it will be possible to predict the general type of gradient for a topographically uniform field, and more experimental work in this direction is needed.

We wish to express our grateful thanks to Dr P. H. Gregory and Mrs M. F. Gregory for their help in many ways during the progress of this work and in the preparation of this paper. Our thanks are also due to Prof. R. Ramanadham for lending the meteorological instruments, to Dr T. V. Avadhani for his help in the calculations, and to Prof. J. Venkateswarlu for his interest in the progress of the work.

REFERENCES

CAMMACK, R. H. (1958). Factors affecting infection gradients from a point source of *Puccinia polysora* in a plot of *Zea mays*. *Ann. appl. Biol.* **46**, 186.

CHAMBERLAIN, A. C. (1956). Aspects of travel and deposition of aerosol and vapour clouds. *A.E.R.E. HP/R*, no. 1261.

GREGORY, P. H. (1945). The dispersion of air-borne spores. *Trans. Brit. mycol. Soc.* **28**, 26.

GREGORY, P. H. (1952). Presidential address: Fungus spores. *Trans. Brit. mycol. Soc.* **35**, 1.

GREGORY, P. H. (1961). *The microbiology of the atmosphere.* London: Leonard Hill.

GREGORY, P. H., LONGHURST, T. J. & SREERAMULU, T. (1961). Diffusion and deposition of air-borne *Lycopodium* and *Ganoderma* spores. *Ann. appl. biol.* **49**, 645.

SCHMIDT, W. (1918). Die Verbreitung von Samen und Blütenstaub durch die Luftbewegung. *Öst. bot. Z.* **67**, 313.

SCHMIDT, W. (1925). Der Massenaustausch in freier Luft und verwandte Erscheinungen. *Probl. kosm. Phys.* **7**, 1.

SCRASE, F. J. (1930). Some characteristics of eddy motion in the atmosphere. *Gt. Brit. Met. Off. Geophys. Mem.* no. 52, p. 3.

STAKMAN, E. C. (1942). The field of extra-mural aerobiology. *Aerobiology*. Publ. Amer. Ass. Adv. Sci. no. 17, 1.

STEPANOV, K. M. (1935). Dissemination of infective diseases of plants by air currents. *Bull. Pl. Prot. Leningr., Ser.* 2, *Phytopathology*, no. 8.

SUTTON, O. G. (1932). A theory of eddy diffusion in the atmosphere. *Proc. roy. Soc.* A, **135**, 143.

SUTTON, O. G. (1947*a*). The problem of diffusion in the lower atmosphere. *Quart. J. roy. met. Soc.* **73**, 257.

SUTTON, O. G. (1947*b*). The theoretical distribution of airborne pollution from factory chimneys. *Quart. J. roy. met. Soc.* **73**, 426.

SUTTON, O. G. (1953). *Micrometeorology*. New York: McGraw-Hill.

SUTTON, O. G. (1955). *Atmospheric turbulence.* London: Methuen.

VAN DER PLANK, J. E. (1948). The relation between the size of fields and the spread of plant diseases into them. Part I. Crowd diseases. *Emp. J. exp. Agric.* **16**, 134.

VAN DER PLANK, J. E. (1949). The relation between the size of fields and the spread of plant diseases into them. Part 2. Diseases caused by fungi with air-borne spores; with a note on horizons of infection. *Emp. J. exp. Agric.* **17**, 18.

WAGGONER, P. E. (1952). The distribution of potato late blight around inoculum sources. *Phytopathology*, **42**, 323.

WILSON, E. E. & BAKER, G. A. (1946). Some aspects of the aerial dissemination of spores, with special reference to conidia of *Sclerotinia laxa*. *J. agric. Res.* **72**, 301.

WOLFENBARGER, D. O. (1946). Dispersion of small organisms. Distance dispersion rates of bacteria, spores, seeds, pollen and insects; incidence rates of diseases and injuries. *Amer. Midl. Nat.* **35**, 1.

Ann. appl. Biol. (1961), **49**, 671–674
Printed in Great Britain

Studies on *Gloeosporium musarum* Cke. & Massee causing storage rots of Jamaican bananas

IV. Large-scale experiments with sodium salicylanilide ('Shirlan WS')

By D. S. MEREDITH
Banana Board Research Department, Kingston, Jamaica, B.W.I.

(*Received* 30 *March* 1961)

SUMMARY

In several commercial-scale experiments, bunches of bananas were treated by immersing for approximately 1 min. in 2% Shirlan WS and then rinsing in water. Reports obtained after ocean transportation and subsequent ripening of fruit in England indicated that this treatment effected poor or no control of fruit-rot caused by *Gloeosporium musarum* Cke. & Massee. Possible reasons for this are discussed in relation to earlier laboratory trials.

INTRODUCTION

In laboratory experiments (Meredith, 1960*a*, *b*, *c*) moderate to good control of banana finger-stalk rot and anthracnose (*Gloeosporium musarum* Cke. & Massee) was obtained when experimentally inoculated fruit was dipped in 1·0–1·5% Shirlan WS. However, as previously observed by Leach (1956), this treatment alone caused phytotoxic effects, particularly on the Lacatan variety of banana. Such effects were prevented by rinsing fruit with water immediately after fungicidal treatment but, as was anticipated, there was some reduction in percentage control (Meredith, 1960*c*). The present paper places on record the results of a series of trials to test this Shirlan WS/washing schedule under commercial conditions.

MATERIALS AND METHODS

All trials were carried out at Bowden wharf, using the Lacatan variety of banana. A 2% aqueous solution of Shirlan WS, plus 0·1% 'Agral LN' wetter, was prepared in a wooden box having a capacity of approximately 400 gallons. Another box containing water only was set up in such a position that bunches of fruit could be transferred to it from the fungicide by rolling over a soft pad. Two other wash boxes contained sodium bisulphate solution (22 lb./100 gallons) and water, respectively.

The sequence of treatments for experimental fruit was as follows: bunches were removed from the truck and immersed for a few seconds in the acid tank to remove unsightly Bordeaux spray residues. A quick rinse in water followed, after which bunches were immersed in the fungicide solution for 0·5–1 min., being agitated continuously; finally there was a water rinse. Control bunches selected randomly from the first water tank received no fungicidal treatment. After allowing excess water to

drain off, the ends of the main-stalk were trimmed; each bunch was enclosed in a plastic bag and a coating of polyethylene polysulphide ('PEPS') was applied to the severed main-stalk ends ('PEPS' treatment is a routine commercial operation for the control of main-stalk rot). Fruit was then stowed on the wharf until ready for loading on to the ship.

Laboratory experience indicated the importance of minimizing the interval between harvesting and treatment with Shirlan WS, there being a marked decrease in percentage control with incubation periods exceeding 24 hr. In the current trials, the average 'age' of fruit at the time of treatment was 16 hr., extremes being approximately 24 and 6 hr., respectively.

On shipboard, conditions under which treated fruit was stored were identical with those for the untreated bulk of the consignment, the temperature inside the hold being lowered to 56–57° F. over a 3-day period. After discharge in the United Kingdom, experimental fruit was distributed to several stores where it ripened at temperatures usually falling within the range 61–70° F. Disease assessments were carried out on fully mature fruit just prior to marketing.

RESULTS

The severity of anthracnose and finger-stalk rot was assessed according to the following arbitrary rating: negligible (0), trace (1), slight (2), medium (3) or heavy (4). A disease index was then calculated:

$$\text{Disease index} = \Sigma\% \text{ number of bunches in category} \times \text{category rating}/4$$

Data obtained from different shipments and at various banana-ripening stores are given in Table 1.

Shirlan WS treatment caused little overall reduction in the severity of finger-stalk rot. In experiments performed during February, March and April, index numbers for control fruit were consistently low, so that even an apparent 40–50% reduction, for instance on 11 April, was of negligible commercial importance. (Previous experience with this disease index indicated that values of less than 15 are of little or no commercial importance; values exceeding 35 are indicative of serious wastage.) In the latter half of the year there was up to 25% reduction of disease in fungicide-treated fruit, but in no instance was the difference statistically significant. Slightly better results were obtained against anthracnose, significant degrees of control being realized in three shipments. However, more consistent control would be necessary to justify routine commercial use of Shirlan WS.

As in previous years (Meredith, 1960*b*) well-marked seasonal variation in severity of *Gloeosporium* rot was evident, index numbers for control fruit in the October–December experiments being higher than those recorded during February, March and April. There was considerable variation between data obtained at different ripening stores, possibly a result of varying experience of individual recorders.

No adverse reports concerning phytotoxic effects were received, thus confirming earlier laboratory experience with this Shirlan WS/washing schedule.

Table 1. *Effect of Shirlan WS on the severity of banana finger-stalk rot and anthracnose: mean disease index numbers obtained from successive shipments of fruit*

Date	Treatment	No. of bunches	Mean disease index no.	
			Finger-stalk rot	Anthracnose
17. ii. 60	Shirlan	894	13·5	6·4
	Control	842	16·8	16·8
14. iii. 60	Shirlan	1100	12·5	8·1
	Control	886	15·5	8·5
23. iii. 60	Shirlan	971	11·8	9·5
	Control	790	10·0	9·2
6. iv. 60	Shirlan	1096	12·8	10·2
	Control	937	13·0	3·9
11. iv. 60	Shirlan	598	6·5	6·1
	Control	505	12·0	12·8
17. x. 60	Shirlan	750	25·1	28·6
	Control	936	28·0	25·8
20. x. 60	Shirlan	840	41·5	19·7*
	Control	826	44·0	32·0
3. xi. 60	Shirlan	563	30·2	16·2*
	Control	771	40·7	30·6
10. xi. 60	Shirlan	767	33·5	15·7
	Control	828	41·5	27·2
24. xi. 60	Shirlan	605	32·5	20·5
	Control	622	35·6	26·0
8. xii. 60	Shirlan	537	37·1	25·1
	Control	535	36·8	28·4
22. xii. 60	Shirlan	831	38·0	41·6
	Control	740	45·3	43·3
30. xii. 60	Shirlan	904	29·8	14·4**
	Control	897	38·5	32·9

* Significant at 5 % level of probability.
** Significant at 1 % level of probability.

DISCUSSION

The reason for these somewhat disappointing results is not known with certainty, but several possible explanations may be considered briefly. First, it might have been that the average time between harvest and treatment with Shirlan WS was too great, with the result that infections established shortly after harvest had developed to a point beyond control. A more detailed investigation on the histology of infection is suggested. Secondly, the period of immersion in the fungicide might have been too short: in laboratory trials it was found that a 2 min. immersion period gave appreciably better control than one of 1 min. or less. Thirdly, it is possible that the post-fungicidal water rinse not only removed excess fungicide from the fruit surface but also some that had penetrated into minor abrasions and other mechanically induced wounds. Of these factors, the second and third would appear most likely to be of importance. Practical difficulties attendant on ensuring a full minute or more for

Shirlan WS treatment, and merely a momentary dip in water, are not inconsiderable, since 30,000–40,000 bunches of fruit have to be processed within about 12 hr. Indeed, on occasions, the planned schedule was not rigidly adhered to. This raises one important point concerning any form of bunch processing in Jamaica, namely that the various operations should be quite simple and capable of being carried out quickly.

In conclusion it may be stated that expectations based on laboratory experience with this Shirlan WS/washing treatment were not borne out under commercial conditions. As has been indicated elsewhere (Meredith, 1961), research on control of banana storage diseases by chemical means is still in its infancy, and it will be necessary to continue the search for a fungicide which is able to provide good control of *Gloeosporium* rot.

The author is indebted to many officers of Messrs Elders and Fyffes Ltd. for making detailed assessments of disease severity in ripening stores, and to Miss V. E. Green for valuable technical assistance. This paper is published by permission of the Banana Board, Kingston, Jamaica, B.W.I.

REFERENCES

Leach, R. (1956). An investigation into the development of banana diseases during transport overseas and in the U.K. ripening rooms. Unpublished Report, Banana Board, Kingston, Jamaica, W.I.

Meredith, D. S. (1960*a*). Studies on *Gloeosporium musarum* Cke. & Massee causing storage rots of Jamaican bananas. I. Anthracnose and its chemical control. *Ann. appl. Biol.* **48**, 279.

Meredith, D. S. (1960*b*). Studies on *Gloeosporium musarum* Cke. & Massee causing storage rots of Jamaican bananas. II. Some factors influencing anthracnose development. *Ann. appl. Biol.* **48**, 518.

Meredith, D. S. (1960*c*). Studies on *Gloeosporium musarum* Cke. & Massee causing storage rots of Jamaican bananas. III. Control with sodium salicylanilide ('Shirlan WS') and nystatin. *Ann. appl. Biol.* **48**, 824.

Meredith, D. S. (1961). Chemical control of transport and storage diseases of bananas. *Trop. Agriculture, Trin.*, **38**, 205.

Ann. appl. Biol. (1961), **49**, 675–691
With 1 plate
Printed in Great Britain

Wilt of lucerne caused by species of *Verticillium*

III. Viability of *V. albo-atrum* carried with lucerne seed; effects of seed dressings and fumigants

By IVOR ISAAC and J. B. HEALE
University College of Swansea

(*Received 23 March* 1961)

SUMMARY

Resting mycelium of *V. albo-atrum* in moribund lucerne remains viable at room temperature for 13 months but is not viable after 2 years, whereas conidia on lucerne seed kept at room temperature fail to germinate after 7 months.

The fungicide, Orthocide (captan) is toxic both to the conidia of *V. albo-atrum* on seed coats of lucerne and to infected plant material carried with seed. Dressed seeds inoculated with *Rhizobium mellelotus* developed into healthy seedlings bearing normal root nodules, although the Orthocide is toxic to the bacterium in culture.

Dressing of seeds with Orthocide, Agrosan-GN (containing mercury) and Fernasan (thiram) *after* inoculation gives slightly higher yields of lucerne than where dressing is done *before* inoculation, but this is not sufficient to recommend a change in the normal commercial procedure of dressing *before* inoculation.

Methyl bromide, used commercially as a fumigant to control *Ditylenchus dipsaci*, has no effect on *Verticillium*. However, allyl alcohol, allyl bromide, chloropicrin and formaldehyde are shown to be useful fumigants to control *Verticillium* carried with seed, since at exposures which kill the resting mycelium and conidia of *V. albo-atrum*, they do not affect the viability of lucerne seed.

INTRODUCTION

Isaac (1957) described the symptoms of the vascular wilt disease of lucerne caused by *Verticillium albo-atrum* and *V. dahliae*, and more recently Isaac & Lloyd (1959) reported on the seasonal cycle of disease, host–parasite relationships and effects of certain seed dressings as a control measure for seed carriage.

The occurrence and spread of lucerne wilt caused by *V. albo-atrum* on the Continent has been reported by Staple (1956) in Denmark; by Wagner (1957) and Kiessig & Haller-Kiessig (1957) in Germany; by Kort & Van Rheenen (1959) in Holland; and by Ribaldi (1959) in Italy.

Since *V. dahliae* has not been reported as a pathogen of lucerne on the Continent, and since it has been found in only two localities in Britain and then causing mild symptoms in occasional plants, this organism is of no economic importance to lucerne and hence in the present paper, except where stated otherwise, the disease caused by *V. albo-atrum* is considered.

VIABILITY OF *VERTICILLIUM ALBO-ATRUM* CARRIED WITH LUCERNE SEED

Isaac (1957) reported that the fungus was carried as resting mycelium with the seed, in or on 'foreign' plant material—probably pieces of pods and/or pedicels which had been broken down in threshing and harvested with the seed—but more recently the present writers have isolated the pathogen directly from the seed of 1959-harvested, English-grown lucerne. The pathogenicity of this isolate to lucerne was established by both soil and wound inoculations and its virulence proved to be equal to that of other lucerne isolates tested at Swansea. This is the first record of the isolation of *V. albo-atrum* from the actual seed coat of lucerne.

It was obvious, therefore, that to effect a control by seed dressing or fumigation, the viability of both the resting mycelium contained in moribund lucerne tissue and of conidia carried on the testa had to be investigated.

Diseased lucerne stems containing the black resting mycelium of the fungus were collected from Norfolk and Lincolnshire during the 1958 harvest and *V. albo-atrum* isolated from them. The material was chopped into $\frac{1}{2}$ in. pieces and, of eleven series of fifty pieces, nine were stored in sterile flasks in the laboratory. The other two series were immediately tested for viable resting mycelium by dividing the stem portions longitudinally and transferring them to sterile moist filter-paper in Petri dishes, incubated at 22·0° C. for 10 days and then examined for the presence of the verticillate conidiophores of *Verticillium*. The stored pieces were similarly tested monthly for 13 months. At the same time, approximately 10 g. of lucerne seeds (Du Puits) were inoculated by shaking them for 1 min. in a concentrated water suspension of conidia, then dried overnight on sterile absorbent paper at room temperature. One hundred seeds were transferred singly to sterile Dox's agar medium in Petri dishes, twenty-five per dish, and incubated at 22·0° C. for 4 days, then examined for *Verticillium*. This test was repeated monthly for 8 months during which the seeds were kept in the laboratory. Similarly, pieces of vermiculite approximating in size to seeds were coated with conidia and examined.

Table 1 indicates that the resting mycelium of *V. albo-atrum* can survive for 13 months in dry moribund tissue kept in the laboratory, retaining the ability to sporulate profusely under damp conditions. However, when some of the same material was retested after a period of 2 years the fungus was no longer viable.

The results for conidia, indicating that they survive for a relatively short period, corroborate the preliminary findings of Isaac & Lloyd (1959). Since conidia would readily develop during damp storage, their importance cannot be ruled out; if they were formed just prior to sowing, a large number of highly viable spores would be distributed with the seed.

Garber & Houston (1959), investigating the inhibitory effect of cotton seed on *V. albo-atrum*, found that the fungus would develop throughout the prune-dextrose medium employed except in the vicinity of the seeds. They found that the fungistatic effect was induced by the testa rather than the embryo. Since, in the present work, *Verticillium* grew profusely on and near the seed testa on Dox's medium and since the conidia survived for approximately the same period on seeds as on vermiculite,

it is clear that no such fungistatic constituents occur in the lucerne seed. Desiccation through the thin walls of the conidia and the paucity of metabolic reserves were probably the main factors responsible for the decline in conidial viability after 4 months. The longer viability of resting mycelium was probably due to the thickening and darkening of its walls, which restricted desiccation.

Since, as noted above, resting mycelium in moribund tissue had lost its viability after 2 years' storage in the laboratory and since Isaac (1957) had shown that lucerne plants could become infected if pods and pedicels containing viable resting mycelium were added to the soil, some stem pieces that had been stored for 2 years were mixed with soil underlying the roots of ten lucerne (Du Puits) seedlings. However, no symptoms developed and the fungus was not isolated. This appears to be conclusive evidence that resting mycelium in dead plant tissues is no longer viable after a period of 2 years and so the chance of dissemination of the disease by such material is negligible.

Table 1. *Viability of resting mycelium contained in moribund pieces of lucerne tissue and of conidia both on lucerne seed and on vermiculite*

(Viability expressed as a percentage of the initial figure before storage.)

Storage period (months)	Resting mycelium	Conidia on seeds	Conidia on vermiculite
1	95	100	100
2	88	100	98
3	88	100	100
4	84	29	99
5	95	10	68
6	—	8	16
7	91	9	0
8	—	0	0
9	81	0	0
11	42	—	—
13	42	—	—

— indicates no test.

SEED DRESSING AS A METHOD OF DISEASE CONTROL

Isaac & Lloyd (1959) found that Agrosan-GN (containing mercury) and Fernasan (containing thiram) were toxic both to conidia of *Verticillium* on lucerne seed and to the mycelium in plant material carried with the seed, and that although they inhibited the growth of *Rhizobium mellelotus* in agar, this did not prevent dressed seed from producing healthy seedlings bearing normal root nodules.

In the present work the effects of a third dressing, Orthocide, both on conidia carried on the seed surface and on resting mycelium in infected material mixed with seed are described. The Orthocide used contained 75·0% captan (*N*-(trichloromethyl-thio)-4-*cyclo*hexane-1, 2-dicarboximide) and 25·0% inert base.

Effect of Orthocide on the viability of conidia. Seeds of lucerne (Du Puits) were coated with conidia as previously described and to four series of 10 g. each in sterile stoppered flasks, 0·01, 0·02, 0·05 and 0·1 g., respectively, of Orthocide were added

giving a weight-for-weight ratio of 0·1, 0·2, 0·5 and 1·0% of dressing to seed, while a further two series were left undressed to act as controls. All series except one of the undressed controls were shaken vigorously for 15 min. to distribute the dressings evenly over the seed. Twenty-four hours later, four replicates, each of twenty-five seeds, were taken from each treatment, transferred to sterile Dox's agar in Petri dishes and incubated at 22·0° C. for 4 days, when they were examined for *Verticillium*. The treatments were stored for 4 weeks and again tested by plating on to agar.

Table 2. *The effects of Orthocide upon the viability of conidia of* Verticillium albo-atrum *adhering to the testa of lucerne* (*Du Puits*)

Seed treatment	Total no. of seeds infected of 100 tested	
	After 1 day	After 4 weeks
Not dressed, unshaken	100	100
Not dressed, shaken	100	100
Dressed with Orthocide at		
0·1% (w/w)	2	0
0·2% (w/w)	1	0
0·5% (w/w)	0	0
1·0% (w/w)	0	0

The results summarized in Table 2 indicate that the Orthocide, at all levels used, markedly reduced the viability of conidia and was lethal to all at a concentration of 0·5% (w/w) after 1 day, and at 0·1% (w/w) after 4 weeks in contact. Vigorous shaking alone (control) had no effect upon conidial viability.

Effect of Orthocide on the viability of the fungus in plant material. Moribund lucerne stems were chopped into pieces approximating in size to lucerne seed and 150–200 of these pieces added to each of four 10 g. samples of seed which were then dressed at 0·1, 0·2, 0·5 and 1·0% (w/w) with Orthocide, while a control sample of seed plus inoculum was left undressed. The five samples were shaken vigorously for 15 min. Twenty-four hours later, 100 pieces of stem were removed from each of the treatments and incubated on moist filter-paper at 22·0° C. for 10 days and then examined for *Verticillium*.

The results in Table 3 indicate that the germination and sporulation of the resting mycelium were effectively reduced at all levels of the dressing employed although the percentage survival was higher than for conidia. This was probably due to the difficulty of penetrating lucerne stem material to reach the resting mycelium and to the thicker walls of the latter. That toxicity resulted was evidence for the effective permeation of the higher concentrations of captan throughout the inoculum.

Effect of Orthocide on stored seeds of lucerne. Four series of 60 g. samples of lucerne (Du Puits) seeds were each dressed with 0·1, 0·2, 0·5 and 1·0% (w/w) respectively, of Orthocide and a series of undressed seed was maintained as a control. The five series were stored in tightly stoppered flasks in the laboratory for 6 months. The water content of a similar sample of seed kept under the same conditions for 6 months remained at approximately 12·0%.

At monthly intervals, four replicates of twenty-five seeds from each treatment were

transferred to moist filter-papers in Petri dishes and incubated at 22·0° C. for 7 days, when they were examined and counts made of the number and types of seedlings developed using the same categories employed by Isaac & Lloyd (1959) as follows:

(1) Healthy seedlings—well-developed radicle, plumule and root hairs.

(2) Abnormal seedlings—with glossy and transparent hypocotyls, swollen root tips and various other malformations.

Table 3. *The effect of Orthocide on material infected with* Verticillium albo-atrum *and mixed with lucerne* (*Du Puits*) *seeds*

Seed treatment	No. of pieces per 100 sampled showing *Verticillium* after dressing and incubation at 22·0° C. for 10 days
Undressed	86
Dressed with Orthocide at	
0·1 % (w/w)	33
0·2 % (w/w)	7
0·5 % (w/w)	0
1·0 % (w/w)	0

(3) Broken seedlings—developed from seeds probably damaged during threshing; frequently consisted of separate radicles and plumules or detached cotyledons.

(4) Dead seeds—had absorbed water but failed to germinate; were soft and easily crushed.

(5) Hard seeds—relatively impervious to water and so remained unswollen for the duration of the 7 days.

The data obtained over the 6 months showed that, as with Agrosan-GN and Fernasan (Isaac & Lloyd, 1959), the viability of lucerne seed was not significantly affected. Since the 0·5% dressing was completely effective against conidia and against resting mycelium in stem tissue, even when these were present in much greater proportions than that to be expected in normal seed consignments, it is suggested that this level could be used commercially as a control, so preventing the introduction of the disease into a new area.

Effects of Orthocide, Agrosan-GN and Fernasan upon the germination and growth of Verticillium albo-atrum *in vitro*. The effects of the three dressings were compared using both the following methods:

(1) Three series, each of four replicate amounts of 0·01 g. of Orthocide, Agrosan-GN and Fernasan, respectively, were placed separately on sterile discs (1 cm. diameter) of absorbent paper in the centre of Dox's agar plates which had been 'seeded' previously with a heavy conidial suspension of *V. albo-atrum*. A similar series without dressing served as a control. The sixteen dishes were incubated at 22·0° C. and after 3 days the inhibition zone from the edge of the disc to the first visible line of conidial germination was measured.

The results indicated that the mercury-containing Agrosan-GN was more inhibitory to the germination of *V. albo-atrum* (inhibition zone: 33·0 mm.) than either Fernasan (inhibition zone: 6·8 mm.) or Orthocide (inhibition zone: 7·4 mm.). Where

germination occurred with the last two dressings, normal hyaline mycelium developed and immediately adjacent to the zones of inhibition, resting mycelium was evident (see Plate, fig. 1). With Agrosan-GN, inhibition was so marked that although germ-tubes were formed near the periphery of the agar plates, neither normal hyaline nor resting mycelium developed.

(2) In this method the three dressings were incorporated into Dox's agar prior to 'flash autoclaving'. The concentrations used were 1, 5, 20 and 50 p.p.m., respectively, for each dressing and a wetting agent—sodium dioctyl sulphosuccinate (S.D.S.)—was added at concentrations of 0·2, 1, 4 and 10 p.p.m., respectively, to facilitate the even dispersal of the dressings throughout the medium. Two further series of normal Dox's medium and Dox's plus S.D.S. were maintained as controls. Four plates were prepared for each concentration of the dressings and these, together with the control plates, were inoculated with discs (3 mm. diameter) of *V. albo-atrum* and incubated at 22·0° C. After 7 days, the diameters of the cultures were measured and the percentage inhibition induced by the dressings was calculated by comparison with the growth on normal Dox's medium.

Table 4. *The effect of Agrosan-GN, Fernasan and Orthocide on the growth of* Verticillium albo-atrum *in vitro*

Dressing	Percentage inhibition* with concentrations of dressing in p.p.m.			
	50	20	5	1
Dox's + Agrosan-GN + S.D.S.	86	45	9	1
Dox's + Fernasan + S.D.S.	8	6	4	0
Dox's + Orthocide + S.D.S.	12	7	4	0
Dox's + S.D.S.	0	0	0	0
Dox's	0	.	.	.

* Percentage inhibition $= (A-B)/A \times 100$, when A = mean diameter of 4 replicate cultures grown on normal Dox's agar for 7 days at 22° C. and B = mean diameter of 4 cultures grown on Dox's medium plus dressing.

The results shown in Table 4 indicate that Agrosan-GN was the most inhibitory and, although some loss of fungicidal activity may have occurred during autoclaving, the results corroborate those obtained by the first method which did not involve heat-sterilization.

Effect of Orthocide on the nodulation of lucerne by Rhizobium mellelotus. To ensure a good stand, the seed coats of lucerne are frequently inoculated with cultures of *R. mellelotus*. Johnson (1951), Ruhloff & Burton (1951) and Mead (1955) have shown that this organism is inhibited by various seed dressings, and although Isaac & Lloyd (1959) also found in preliminary experiments that both Agrosan-GN and Fernasan were toxic to *Rhizobium* in agar culture, they reported that dressed seeds which had been inoculated with the bacterium developed into healthy seedlings bearing normal root-nodules.

To test the effect of Orthocide upon the viability of *R. mellelotus* on the seed coat, the dressing was added to two lots of seeds, one *after* inoculation, the other *before* inoculation. 150 ml. of sterilized skim milk were inoculated with *R. mellelotus* (strain

AH2) obtained from Rothamsted Experimental Station and incubated at 22·0 °C. for 7 days, after which the culture was thoroughly mixed with a sample of lucerne (Du Puits) seed. The liquid was then decanted and the seeds were allowed to dry overnight at room temperature. Orthocide was added to four 1 g. samples of these inoculated seeds at 0·1, 0·2, 0·5 and 1·0% (w/w), respectively, while four similar samples of uninoculated seed were dressed with corresponding levels of the chemical and then treated with the bacterium as described above. Suitable control series of dressed and uninoculated, undressed and uninoculated, and undressed and inoculated seeds were maintained. Fifty seeds from each treatment were sown in sterile soil (autoclaved at 15 lb./sq.in. for 3 hr.) in boxes. After 2 months, counts of established plants were made and dry-weight yields calculated; a number of plants in each treatment were examined for the presence of well-developed red nodules since only these are beneficial to the plant. The results are summarized in Table 5.

Table 5. *The effect of Orthocide on the subsequent development of lucerne seedlings from dressed seed inoculated with* Rhizobium mellelotus

Treatment	Presence (+) or absence (−) of red root nodules	No. of seedlings established out of 50 tested	Mean dry wt. per plant in mg. with standard error
Uninoculated and undressed	−	47	248± 7·4
Inoculated with *R. mellelotus* and undressed	+	46	370±13·4
Inoculated with *R. mellelotus before* dressing with Orthocide			
0·1 % (w/w)	+	43	381± 8·8
0·2 % (w/w)	+	34	
0·5 % (w/w)	+	43	
1·0 % (w/w)	+	43	
Inoculated with *R. mellelotus after* dressing with Orthocide			
0·1 % (w/w)	+	45	323±11·4
0·2 % (w/w)	+	41	
0·5 % (w/w)	+	46	
1·0 % (w/w)	+	43	
Uninoculated seed dressed with Orthocide			
0·1 % (w/w)	−	48	219±9·2
0·2 % (w/w)	−	46	
0·5 % (w/w)	−	46	
1·0 % (w/w)	−	46	

Good nodulation occurred on all plants grown from inoculated seed irrespective both of the relative timing, and of the levels of dressing used, but it was poor on plants grown from uninoculated seed. These observations are in accord with the earlier report of Isaac & Lloyd (1959).

Although there were no significant differences between the numbers of established plants in the various treatments, the results indicate that the inoculated seeds yielded a greater dry weight of seedlings than did the uninoculated, and that dressing with Orthocide *after* inoculation resulted in slightly higher yield than that obtained when dressing occurred *before* inoculation.

Effect of Agrosan-GN, Fernasan and Orthocide on Rhizobium mellelotus *in vitro*. Since Isaac & Lloyd (1959) reported that both Agrosan-GN and Fernasan were

inhibitory to *Rhizobium* in culture, the effect of Orthocide was compared with that of these two dressings. A suspension of *Rhizobium* was spread over the surface of plates of yeast–water medium. Four replicate amounts, each of 0·01 g. of each of the three dressings, were placed centrally on twelve of the plates as described previously, while a series of discs without dressing on four plates were maintained as controls. All the dishes were incubated at 22·0° C. and examined after 5 days. As well as measuring the inhibition zones, attempts were made to test the viability of the *Rhizobium* by transferring pieces of agar plus the bacteria from these zones to fresh medium and thus establishing whether the dressing was bactericidal or bacteristatic.

The results obtained indicated that the mercury-containing Agrosan-GN was most toxic to *Rhizobium* giving a zone of 34·0 mm. as contrasted with 10·0 mm. for both Orthocide and Fernasan. The evidence from the attempts at subculturing showed that Agrosan-GN was invariably bactericidal, whereas the other two dressings were generally bacteristatic; Orthocide, however, was bactericidal at the highest concentrations near the disc. Mead (1955) showed that mercurial compounds are more liable to injure the bacterial inoculum but although Agrosan-GN and, to a lesser extent, Fernasan and Orthocide exhibit this adverse effect in *in vitro* tests, the results of Isaac & Lloyd (1959) and the present work clearly show that not one of these three dressings reduces the concentration of *Rhizobium* on the seed coat below that required to produce nodulation.

Effect of Agrosan-GN, Fernasan and Orthocide on the first-year stand of lucerne. A field trial was conducted with the assistance of British Crop Driers Ltd., Lincolnshire, to make a comparison of the effects of these dressings on the first-year stand of lucerne. Twelve $\frac{1}{4}$-acre plots were sown with French (Du Puits) seed that had been treated as follows: three series of seed samples were inoculated with *Rhizobium mellelotus* 24 hr. *before* dressing with 0·5% (w/w) of Agrosan-GN, Fernasan and Orthocide, respectively, while another three series, to act as controls, were left undressed after inoculation. Each of the six series was sown in one of six plots, a control treatment being adjacent to each of the dressed plots. A further three series of seed were inoculated with the bacterium 24 hr. *after* dressing with 0·5 % (w/w) of the three chemicals, respectively; three series of inoculated seeds that had not been dressed again acted as controls. After a further 24 hr. each of these six series was sown in one of six further plots with a control undressed treatment being adjacent to each of the dressed plots. Sowing occurred in April and harvesting 18 weeks later when the dry-weight yields per acre for the various treatments were estimated.

The results shown in Table 6 indicate that inoculation *before* dressing with Agrosan-GN, Fernasan and Orthocide resulted in slightly larger yields of lucerne as contrasted with the undressed control plots. This slight improvement in crop growth was most noticeable immediately before harvesting in August, probably because of the presence of large open weed patches in the control plots and their absence in the dressed plots. This effect may have been due, as suggested by Mead (1955) to the protection against fungal attack given by the adherence of a layer of dressing to the germinated seed, thereby allowing a more rapid development of the seedlings.

When inoculation occurred *after* dressing there were no differences between the dressed and undressed control plots, possibly due to the considerable dilution of the

dressing that occurred during the inoculation procedure. This latter result is in accord with the findings of Tyler, Murphy & McDonald (1956) and of Athow (1957).

In view of the fact that the normal commercial procedure would involve inoculation after dressing, the former being carried out within 48 hours of sowing, the slight improvement in the first-year stand obtained when these treatments were reversed is probably of no economic importance.

Table 6. *The effect of Agrosan-GN, Fernasan and Orthocide on the dry weight yield of first-year stands of lucerne*

Seed treatment with the dressings at 0·5 % (w/w)	Dry wt. yield (cwt./acre)	
	Dressed seed	Undressed seed
Inoculation *before* dressing		
Agrosan-GN	24·6	21·2
Fernasan	27·3	24·2
Orthocide	23·6	20·6
Mean	25·2	22·0
Inoculation *after* dressing		
Agrosan-GN	23·5	24·6
Fernasan	24·2	24·7
Orthocide	28·0	27·9
Mean	25·2	25·7

However, it has been established that these three dressings—Agrosan-GN, Fernasan and Orthocide—eliminate seed carriage while at the same time they are 'safe' in use, since they have no deleterious effects on the germination, nodulation or subsequent stand and yield of lucerne.

SEED FUMIGATION AS A METHOD OF DISEASE CONTROL

Fumigation with methyl bromide has become a standard treatment of lucerne seed to control *Ditylenchus dipsaci* (stem eelworm). Since Lloyd (1959) found that commercial fumigation with methyl bromide of seeds artificially inoculated with *Verticillium* did not adversely affect the fungus, further experiments were conducted by the present writers to investigate the possibility of controlling seed-carriage by the use of other volatile fumigants.

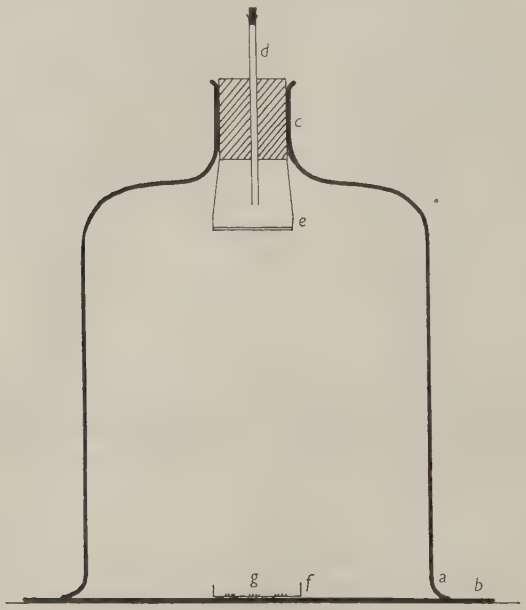
(1) *Agar disc screening test*

This technique was adopted to test the effects of the vapours of allyl bromide, allyl alcohol, allyl isothiocyanate, allyl chloride, chloropicrin, formaldehyde (source used was formalin, 36%, w/v), ethylene oxide, propylene oxide, ethylene dibromide, ethylene dichloride, methyl bromide, D.D.-mixture, 1,2-dichloropropane, 1,3-dichloropropene and carbon disulphide on both *V. albo-atrum* and *V. dahliae* in pure culture.

The apparatus used consisted of a modified bell-jar (Text-fig. 1) with the base (*a*) greased to provide an airtight seal on a ground-glass plate (*b*). The neck of the bell-jar was sealed with a rubber stopper (*c*) through which passed a narrow capillary

tube (*d*), with its upper end closed by a small rubber stopper and its lower open end just above an open Petri dish (*e*) suspended from the neck of the bell-jar to retain the liquid fumigant.

1·0 ml. of the fumigant under test was delivered through the tube into the retaining dish and the capillary stopper replaced. Part of the fumigant volatilized to produce a saturated atmosphere in the chamber and the remainder served as a reservoir to maintain saturation after the opening of the chamber for changes of treatment. Four replicate agar discs (3 mm. in diameter) were taken from each of four isolates of



Text-fig. 1. Apparatus used for fumigation of cultures (*g*) of *V. albo-atrum* and *V. dahliae*

Verticillium from lucerne—two of *V. albo-atrum* and two of *V. dahliae*. Each disc consisted of resting structures (either the resting mycelium of *V. albo-atrum* or the microsclerotia of *V. dahliae*), hyaline mycelium and conidia. The sixteen discs (*g*) were placed in a Petri dish (*f*) on the glass plate (*b*). Fifteen minutes were allowed after the introduction of the fumigant before the treatments were placed in the chamber and again between treatments. After exposure, the discs were transferred to sterile Dox's agar and incubated at 22·0° C. for 10 days. Control unfumigated discs were similarly incubated and examined.

A preliminary test showed that after exposure for 30 and 60 min. to allyl isothiocyanate, allyl chloride, propylene oxide, ethylene dibromide, ethylene dichloride, methyl bromide, D.D.-mixture, 1,2-dichloropropane and carbon disulphide, all the discs survived and germinated. With 1,3-dichloropropane, after 60 min. exposure none

of the discs germinated: however, after 30 min., discs of *V. dahliae* survived but not those of *V. albo-atrum*. The vapours of allyl bromide, allyl alcohol, chloropicrin, formaldehyde and ethylene oxide were toxic to all the discs at both exposure periods.

These results for methyl bromide corroborate those of Lloyd (1959), showing that this nematocide is of no value in eliminating *Verticillium* from contaminated lucerne seed. Parris (1945) and Newhall & Lear (1948) also showed the non-activity of D.D.-mixture and of its component, 1,2-dichloropropane, while Lange (1946) and Newhall & Lear (1948) showed that 1,3-dichloropropene (the other main constituent of D.D.-mixture) showed some fungicidal effect. Horsfall (1956) and Schmitt (1949) described carbon disulphide and allyl chloride as relatively poor fungicides and almost all workers using ethylene dibromide have concluded that it possesses low fungitoxicity (Schmitt, 1949). Walker, Morell & Foster (1937) demonstrated the toxicity of allyl isothiocyanate to fungi: however, in the present experiments, all the treated discs survived but showed delayed germination. Ark (1947) found that propylene oxide was toxic to *Verticillium* sp. but this was not shown in the present experiments.

Although there was undoubtedly much loss of the highly volatile ethylene oxide (boiling-point 10·0° C.) sufficient quantities remained to produce a toxic effect (see Whelton, Phaff, Mrak & Fisher (1946) and Hansen & Snyder (1947)). Allyl alcohol, which proved so effective in the present work, has been used for the control of 'damping off' of tomato seed beds by Overman & Burgis (1956). The observed fungitoxicity of allyl bromide and chloropicrin to *Verticillium* was also shown by Pratt (1958), while the effectiveness of formaldehyde against *Verticillium* sp. in soil has been reported by Keyworth & Davies (1940) and Jacks (1948).

Lloyd (1959) found that whereas chloropicrin at 1, 2 or 3 ml. per bushel of inoculated soil completely eradicated both *V. albo-atrum* and *V. dahliae*, allyl alcohol, allyl bromide and allyl isothiocyanate, at similar concentrations, merely reduced the numbers of colonies developing on soil dilution plates. Methyl bromide under these conditions had no effect.

Allyl alcohol, allyl bromide, chloropicrin and formaldehyde were used in further tests with shorter exposure periods. Discs containing the following were prepared:

(1) Resting mycelium, hyaline mycelium and conidia of *V. albo-atrum*.

(2) Microsclerotia, hyaline mycelium and conidia of *V. dahliae*.

(3) Hyaline mycelium and conidia of *V. albo-atrum*—taken from a hyaline saltant.

(4) Hyaline mycelium and conidia of *V. dahliae*—taken from a hyaline saltant.

(5) Conidia of *V. albo-atrum*—taken from a concentrated spore suspension in Dox's agar medium. Since in preliminary experiments the conidia of both *V. albo-atrum* and *V. dahliae* reacted identically, only those of the former were tested.

(6) Germinating conidia of *V. albo-atrum*—taken from agar in which conidia had just germinated.

After fumigation for varying periods up to 22 min. as described previously, all the discs were transferred to Dox's medium and incubated at 22·0° C. On the 3rd, 5th, 7th and 10th days the numbers of germinated discs were recorded and the colony diameters were measured (if germination had not occurred, the discs were recorded as 3 mm. for the calculation of the mean value). Since the relative growth rates of all

the fumigated discs were similar, only the numbers of discs germinating (of four replicates) after 10 days are shown in Table 7.

Allyl alcohol was the most toxic fumigant, all types of discs failing to germinate after only 4 min. exposure. With formaldehyde after 8 min. exposure, no germination occurred, while with chloropicrin and allyl bromide all the discs failed to survive after 22 min.

The percentage numbers of discs germinating throughout the period of 10 days are given in Table 8.

Table 7. *The effects of allyl alcohol, allyl bromide, chloropicrin and formaldehyde upon* Verticillium albo-atrum (*V.aa.*) *and* V. dahliae (*V.d.*)

(Records taken 10 days after exposure to the fumigants.)

	Number of discs germinated after exposure periods (in min.) of													
Fumigant and type of disc	1	2	3	4	5	6	8	10	12	14	16	18	20	22
Allyl alcohol														
1. Resting mycelium, V.aa.	4	.	.	.	.	.	.	.	.	.	.	.	.	.
2. Microsclerotia, V.d.	4	2	1	.	.	.	.	.	.	.	.	.	.	.
3. Hyaline mycelium, V.aa.	4	.	.	.	.	.	.	.	.	.	.	.	.	.
4. Hyaline mycelium, V.d.	4	.	.	.	.	.	.	.	.	.	.	.	.	.
5. Conidia, V.aa.	3	.	.	.	.	.	.	.	.	.	.	.	.	.
6. Germinated conidia, V.aa.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Allyl bromide														
1. Resting mycelium, V.aa.	4	4	4	4	4	4	4	4	4	4	3	2	.	.
2. Microsclerotia, V.d.	4	4	4	4	4	4	4	4	2	2	1	.	.	.
3. Hyaline mycelium, V.aa.	4	4	4	4	4	4	4	4	4	4	4	2	1	.
4. Hyaline mycelium, V.d.	4	4	4	4	4	4	3	2	1	1	.	.	.	.
5. Conidia, V.aa.	4	.	.	.	.	.	.	.	.	.	.	.	.	.
6. Germinated conidia, V.aa.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chloropicrin														
1. Resting mycelium, V.aa.	4	4	4	4	4	4	2	.	.	.	.	.	.	.
2. Microsclerotia, V.d.	4	4	4	4	4	4	4	4	4	4	4	4	4	.
3. Hyaline mycelium, V.aa.	4	4	4	4	4	4	3	.	.	.	.	.	.	.
4. Hyaline mycelium, V.d.	4	4	4	4	4	4	.	.	.	.	.	.	.	.
5. Conidia, V.aa.	4	4	4	4	4	4	.	.	.	.	.	.	.	.
6. Germinated conidia, V.aa.	4	3	1	.	.	.	.	.	.	.	.	.	.	.
Formaldehyde														
1. Resting mycelium, V.aa.	4	4	4	4	3	3	.	.	.	.	.	.	.	.
2. Microsclerotia, V.d.	4	4	4	3	2	.	.	.	.	.	.	.	.	.
3. Hyaline mycelium, V.aa.	4	4	4	4	4	4	.	.	.	.	.	.	.	.
4. Hyaline mycelium, V.d.	4	4	4	2	.	.	.	.	.	.	.	.	.	.
5. Conidia, V.aa.	4	4	4	.	.	.	.	.	.	.	.	.	.	.
6. Germinated conidia, V.aa.	4	4	.	.	.	.	.	.	.	.	.	.	.	.

The results indicate that allyl alcohol induced a greater percentage delay period than the other three fumigants, as 67% of the discs did not germinate until after 5 days' incubation whereas control discs germinated within 2 days.

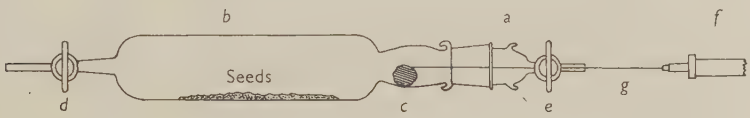
The survival rate of discs containing the microsclerotia of *V. dahliae* as shown in Table 7 is invariably higher than for discs containing hyaline mycelium of the same species. However, there are no apparent differences between the survival of resting mycelium and hyaline mycelium of *V. albo-atrum*, probably because the 'ropes' of hyphae in the hyaline variant have a similar effect to the thickened and pigmented walls of the resting mycelium in reducing the rate of permeation of the fumigant.

The differences in survival period of ungerminated and germinated conidia suggest that the germ tube wall is less resistant to permeation than the wall of the conidium and that the protoplasm of the spore becomes more susceptible to toxicants on germination.

Table 8. *The effect of allyl alcohol, allyl bromide, chloropicrin and formaldehyde on the percentage numbers of discs of* Verticillium albo-atrum *and* V. dahliae *germinating during the* 10*-day period of observation*

	Percentage numbers of discs germinating during the days			
Fumigant	1–3	3–5	5–7	7–10
Allyl alcohol	19	14	58	9
Allyl bromide	65	12	18	5
Chloropicrin	57	29	14	0
Formaldehyde	62	26	12	0

All discs that did germinate after fumigation had done so by the end of the 10 days. All control discs germinated within 2 days of being transferred to fresh medium.



Text-fig. 2. Apparatus (Turtle chamber and micrometer syringe) used for fumigation of infected seed samples.

(2) *Seed fumigation with allyl alcohol, allyl bromide, chloropicrin, and formaldehyde*

The effectiveness of these four fumigants against *V. albo-atrum* in lucerne seed samples was next investigated. The fumigation was effected in glass vessels of approximately 1 l. capacity, known as 'Turtle chambers' (Lubatti & Smith, 1948) (Text-fig. 2). After the removal of the end-fitting (*a*), 10 g. of lucerne (Du Puits) seed were placed in the glass reservoir (*b*) and a small pad of non-absorbent cotton-wool was transferred to the dosing recess (*c*). The end-fitting was replaced, taps (*d*) and (*e*) closed and the chamber left overnight in a constant-temperature room at 25·0° C. to equilibriate the humidity of the air in the vessel with the moisture level of the seeds. The following morning the chamber was dosed with 0·5 ml. of liquid fumigant using an Agla Micrometer Syringe (*f*) adapted with a long metal capillary (*g*) to pass through tap *e* to the dosing pad (*c*). After the required exposure period, the end-fitting (*a*) and the cotton-wool pad were removed, *a* was replaced, taps *d* and *e* opened and the fumigant evacuated for a standard period of 5 min. by means of a suction pump. The seeds were subsequently removed to sterile Petri dishes for examination.

(*a*) *Fumigation of seed samples inoculated with conidia.* Seeds inoculated with conidia of *V. albo-atrum* as described previously, were treated with each of the four fumigants for varying exposures and subsequently four replicate sets, each of twenty-five seeds, were removed from each of the treated samples to Dox's agar and incubated for 4 days at 22·0° C. when they were examined for *Verticillium*. Suitable controls (viz. seed plus conidia, not fumigated) were maintained.

The results indicate that allyl bromide and chloropicrin were the most toxic fumigants to conidia.

(*b*) *Fumigation of seed samples mixed with moribund pieces of lucerne containing resting mycelium.* Four series each of 10 g. of lucerne seed (Du Puits), mixed with approximately 150–200 pieces of moribund lucerne (approximating in size to the seed), were treated separately with each of the four fumigants for varying periods of exposure.

Table 9. *The effects of allyl alcohol, allyl bromide, chloropicrin and formaldehyde upon the viability of conidia of* Verticillium albo-atrum *carried on lucerne seed*

Exposure period in turtle chamber (min.)	Number of seeds out of 100 sampled carrying viable conidia after fumigation			
	Allyl alcohol	Allyl bromide	Chloropicrin	Formaldehyde
10	100	.	6	74
20	97	.	.	72
30	52	.	.	71
40	42	.	.	70
50	2	.	.	70
60	.	.	.	61
70	.	.	.	45
90	.	.	.	14
120	.	.	.	6
150	.	.	.	3
180	.	.	.	2
210	.	.	.	.

All the control seeds carried viable conidia.

Table 10. *The effects of allyl alcohol, allyl bromide, chloropicrin and formaldehyde upon resting mycelium of* Verticillium albo-atrum *in moribund lucerne carried with lucerne seed*

Exposure period in turtle chamber (min.)	Number of pieces of 100 sampled carrying viable resting mycelium after fumigation			
	Allyl alcohol	Allyl bromide	Chloropicrin	Formaldehyde
30	30	16	68	45
60	10	6	60	36
90	.	8	40	10
120	.	.	24	4
180	.	.	20	2
240	.	.	12	.
300	.	.	8	.
360	.	.	2	.
480	.	.	.	.
600	.	.	.	.

The control pieces showed 85–95 % viability.

After fumigation, 100 pieces were separated from the seeds and incubated on moist filter-paper at 22·0° C. for 10 days, after which they were examined for *Verticillium*. Control pieces that had not been fumigated were similarly incubated and examined.

The figures shown in Tables 9 and 10, with the exception of those for formaldehyde, indicate that higher exposure periods were necessary to kill the resting mycelium than

the conidia. This result is similar to that obtained with all four fumigants in both the agar-disc test and seed dressing experiments discussed previously.

Chloropicrin and allyl bromide were most effective against conidia, while allyl alcohol and allyl bromide were most toxic to resting mycelium, and it is suggested that these differences were due to a differential permeability through the dry lucerne tissue, as contrasted with direct permeation through the conidial wall. There are no marked similarities in the relative toxicity of these four fumigants shown in Tables 9 and 10 and those obtained in the agar-disc test, and it is suggested that this again was due to differential permeability (see Oster & Golden, 1947).

Table 11. *The effects of allyl alcohol, allyl bromide, chloropicrin and formaldehyde on the viability of lucerne seed of three levels of moisture content*

Fumigant and exposure period (hr.)	Percentage moisture content of seed	Immediate viability	Stored at 15° C.			Stored at room temperatures		
			3 months	6 months	8 months*	3 months	6 months	8 months*
Control (unfumigated)	8·9	96·5	98·0	96·0	90·0	98·0	90·0	83·0
	14·9	98·5	94·0	95·0	83·0	93·0	81·0	78·0
	16·4	95·5	93·0	90·0	77·0	94·0	78·0	70·0
Allyl alcohol, 1½ hr.	8·9	98·0	100·0	95·0	81·0	98·0	97·0	89·0
	14·9	93·0	96·0	94·0	74·0	90·0	85·0	79·0
	16·4	93·0	91·0	84·0	71·0	87·0	77·0	70·0
Allyl bromide, 2 hr.	8·9	97·0	96·0	96·0	83·0	95·0	90·0	82·0
	14·9	82·0	72·0	78·0	72·0	79·0	67·0	79·0
	16·4	81·5	73·0	80·0	75·0	75·0	68·0	71·0
Chloropicrin, 8 hr.	8·9	92·0	90·0	92·0	88·0	92·0	78·0	78·0
	14·9	76·0	79·0	78·0	76·0	76·0	72·0	70·0
	16·4	75·5	74·0	72·0	68·0	74·0	76·0	70·0
Formaldehyde, 3 hr.	8·9	99·5	97·0	96·0	89·0	94·0	92·0	82·0
	14·9	97·0	96·0	95·0	78·0	91·0	80·0	72·0
	16·4	97·5	95·0	81·0	73·0	90·0	81·0	75·0

* The 8-month figures refer to the soil establishment test.

(*c*) *Effect of fumigation on the viability of lucerne seed.* Lucerne seed (Du Puits) was treated with each of the four fumigants using the minimum exposure period that was toxic to both conidia and resting mycelium—allyl alcohol 1½ hr., allyl bromide 2 hr., chloropicrin 8 hr. and formaldehyde 3 hr. Seeds of moisture content 8·9, 14·9 and 16·4% were fumigated and tested for viability by placing 200 seeds from each treatment on moist filter-paper in Petri dishes kept at 22·0° C. for 7 days. Of the remaining seeds, half was stored at 15·0° C. and half at room temperature (approximately 22·0° C.) and viability tests were again made after 3 and 6 months. At the end of the 8 months' storage, 100 seeds from each treatment were sown in John Innes' Seed Compost and counts made of established plants after a further 6 weeks. Unfumigated seeds of each moisture level were similarly tested for viability and soil establishment, thus functioning as controls.

The results of the filter-paper tests shown in Table 11 indicate that the viability of control seeds gradually decreased over the 6 months. While this was negligible in seed samples of the lowest moisture content stored at 15·0° C., it was most marked in

samples of higher moisture levels kept at room temperature. The fumigated seeds also showed this trend; those treated with allyl bromide and chloropicrin invariably had markedly lower percentage germination figures than the control seeds, particularly those of 14·9 and 16·4% moisture content.

The results of the soil test indicate that although fumigation followed by storage at 15·0° C. was slightly harmful, the establishment of seedlings from seeds kept at room temperature was similar to that of the corresponding controls. Since the results obtained in the filter-paper test tended to over-emphasize the effects of fumigation, it is suggested that an assessment of established seedlings from fumigated seed is important in analysing viability after treatment.

As the moisture content of imported lucerne seed has been shown to vary from 9 to 16% with the average near 12% (personal communication, Dr Blackith, Imperial College Field Station, Ascot), it is suggested that any one of these four fumigants could be used to control the resting mycelium and/or conidia of *V. albo-atrum* at dosages which would not significantly lower the viability of seed of average moisture contents. The fumigation of seed of high moisture content, particularly with allyl bromide or chloropicrin, might result in a slight lowering of germination capacity; in this event formaldehyde or allyl alcohol might be more safely employed.

The effectiveness of these fumigants against *Ditylenchus dipsaci* (stem eelworm) will be the subject of investigation elsewhere.

The writers wish to thank the Agricultural Research Council for financial assistance to carry out this investigation, Miss D. John for her technical assistance, and British Crop Driers Ltd., Lincoln, and British Field Products Ltd., Norfolk, for facilities provided.

REFERENCES

ARK, P. A. (1947). Disinfecting power of propylene oxide and propylene chloride in relation to phytopathogenic bacteria and fungi. *Phytopathology*, **37**, 842.

ATHOW, K. L. (1957). The effect of seed treatment on seedling stand and forage yield of alfalfa and red clover. *Phytopathology*, **47**, 504.

GARBER, R. H. & HOUSTON, B. R. (1959). An inhibitor of *V. albo-atrum* in cotton seed. *Phytopathology*, **49**, 449.

HANSEN, H. N. & SNYDER, W. C. (1947). Gaseous sterilization of biological materials for use as culture media. *Phytopathology*, **37**, 369.

HORSFALL, J. G. (1956). *Principles of fungicidal action*. Watham, Mass., U.S.A. *Chronica Botanica Co.*

ISAAC, I. (1957). Wilt of lucerne caused by species of *Verticillium*. *Ann. appl. Biol.* **45**, 550.

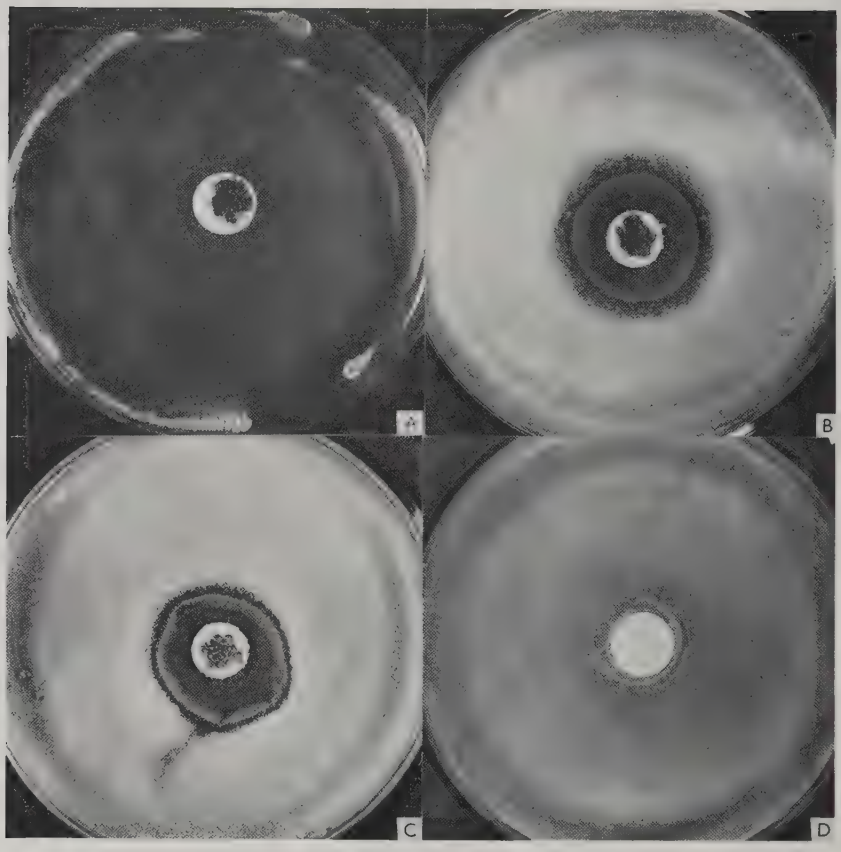
ISAAC, I. & LLOYD, A. T. E. (1959). Wilt of lucerne caused by species of *Verticillium*. II. Seasonal cycle of disease; Range of pathogenicity; Host–parasite relations; Effects of seed dressings. *Ann. appl. Biol.* **47**, 673.

JACKS, H. (1948). Soil disinfection. VIII. Chemical control of *Verticillium* wilt of tomatoes (*V. dahliae*, Kleb.). *N.Z. J. Sci. Tech.* **30**, 118.

JOHNSON, H. W. (1951). Soybean seed treatment. *Soybean Dig.* **11**, 17.

KEYWORTH, W. G. & DAVIES, D. L. G. (1950). Hop diseases. A report of the preliminary investigations carried out in 1938 and 1939 on certain hop diseases. Reprinted from *J. Kent. Br. N.F.U.* Dec. 1939 and Jan. 1940.

KIESSIG, R. & HALLER-KIESSIG, RENATE (1957). Beitrage zur Kenntnis einer infektiösen Welkekrankheit der Luzerne (*V. albo-atrum* R. et B.). *Phytopath. Z.* **31**, 185.


A
B
C
D

Kort, J. & Van Rheenen, H. A. (1959). Enkele Waarnemingen over het optreden van de verwelingsziekte bij Luzerne in Zeeland in 1957. *T. Pl. Ziekten*, **65**, 147.

Lange, W. G. Jr (1946). The use of two new soil fumigants, D.D. and E.D.B., for wireworm control in California. Univ. of California. (Processed.)

Lloyd, A. T. E. (1959). Some investigations into plant diseases caused by species of *Verticillium*. M.Sc. Thesis. University of Wales.

Lubatti, O. F. & Smith, B. (1948). Determination of fumigants. XIX. Sorption of methyl-bromide by onion seed. *J. Soc. Chem. Ind.* **67**, 297.

Mead, H. W. (1955). The effects of fungicides on seedling diseases of legumes and grasses in Saskatchewan. *Canad. J. Agric. Sci.* **35**, 329.

Newhall, A. G. & Lear, B. (1948). Soil fumigation for fungus control with methyl bromide. *Phytopathology*, **38**, 38.

Oster, K. A. & Golden, M. J. (1947). Studies on alcohol-soluble fungistatic and fungicidal compounds. 1. Evaluation of fungistatic laboratory test methods. *J. Amer. Pharm. Ass. Sci. Ed.* **36**, 283.

Overman, A. J. & Burgis, D. S. (1956). Allyl alcohol as a soil fungicide. *Phytopathology*, **46**, 532.

Parris, G. K. (1945). The nematocidal and fungicidal value of D.D. mixture and other soil fumigants. *Phytopathology*, **35**, 771.

Pratt, M. J. (1958). Occurrence, behaviour and control of *Verticillium albo-atrum* R. et B. in small fruits. *Diss. Abstr.* **18**, 36.

Ribaldi, M. (1959). Aspetti e problemi attuali del deperimento dei Medicai italiani. (Primi risultati di un quadrennio di ricerche). *Ann. Fac. Agr. Perugia*, **14**, 12.

Ruhloff, Marcia & Burton, J. C. (1951). Compatability of *Rhizobia* with seed protectants. *Soil Sci.* **72**, 283.

Schmitt, G. G. (1949). Comparison of volatile soil fungicides. *Phytopathology*, **39**, 21.

Staple, C. (1956). Nogle Lucernesygdomme. *Landbonyt*, 1956.

Tyler, L. J., Murphy, R. P. & Macdonald, H. A. (1956). Effect of seed treatment on seedling stands and on hay yields of forage legumes and grasses. *Phytopathology*, **46**, 37.

Wagner, F. (1957). Zum Problem der Lucernewelke auf grund mykologischer Untersuchung der Wurzeln. *Pflanzenschutz*, **9**, 109.

Walker, J. C., Morell, S. & Foster, H. H. (1937). Toxicity of mustard oils and related sulphur compounds to certain fungi. *Amer. J. Bot.* **24**, 536.

Whelton, R., Phaff, H. J., Mrak, E. M. & Fisher, C. D. (1946). Control of microbiological food spoilage by fumigation with epoxides. *Food. Indus.* **18**, 23.

EXPLANATION OF PLATE

The effect of Agrosan-GN (A), Fernasan (B) and Orthocide (C), placed on central discs of filter-paper, upon the germination of conidia of *V. albo-atrum* as contrasted with control (D). Note the inhibition zones and the stimulation to formation of resting mycelium adjacent to the zone induced by Fernasan and Orthocide.

Ann. appl. Biol. (1961), **49**, 692–703
With 1 plate
Printed in Great Britain

The effect of vernalization on embryos of field-resistant wheat varieties infected by loose smut (*Ustilago tritici*)

By P. G. MANTLE*
Botany Department, Imperial College, London, S.W. 7

(*Received* 23 *March* 1961)

SUMMARY

The occurrence of an incompatible host/parasite reaction between winter wheat varieties infected with three Cambridge loose smut races was observed following vernalization of inoculated seed. This led to a re-investigation of certain embryo-susceptible field-resistant combinations and the incompatibility reaction was found to be usual. Other unusual reactions were also observed. Whereas it was previously thought that loose smut mycelium failed to penetrate the plumular bud of embryo-susceptible field-resistant wheat varieties, it is shown that in fact the plumular bud may be infected with a subsequent serious effect on the host's development. Consequently a modified scheme for determining varietal reaction to loose smut infection is suggested.

INTRODUCTION

The investigation of an abnormal seedling reaction of Kota wheat, formerly attributed to hypersensitivity (Oort, 1944), revealed that it was due to epicotyl infection by loose smut mycelium (Mantle, 1961*a*). This was termed an incompatibility reaction. No new meaning was intended to be read into this term whose usage in this context simply inferred a discordance between host and parasite in certain combinations. However, in each sample of seed, inoculated with a race with which the host reacted to show the abnormal symptoms, there were a number of embryos in which the pathogen was confined to the scutellum.

Batts & Jeater (1958*b*) investigated the fungal distribution in winter wheat varieties which, when embryo-tested, appeared to be susceptible, but when grown in the field grew quite normally and produced uninfected ears. They found that in these combinations the mycelium was confined to the scutellum. Popp (1959) confirmed that the plumular buds of field-resistant varieties were not infected, although the scutella might be infected.

The reason for the failure of the pathogen to penetrate from the scutellum to the plumular bud is not known. Therefore, in the present investigation an attempt was made to encourage the mycelium within the scutellum to infect the plumular bud. If this proved possible, then the plant would probably either show an incompatibility reaction to infection or produce a smutted ear after growing normally.

Oort (1944) only observed 'hypersensitive' symptoms clearly in greenhouse tests.

* Now at Biochemistry Department, Imperial College, London, S.W. 7.

In the field such seedlings rarely emerged. Thus to observe the abnormal reaction the seed would have to be sown under good growing conditions, and for this to be practicable on a large scale a field plot would be required. To obtain good growing conditions, similar to an unheated greenhouse in summer, the seed could not be sown before late April.

Most of the embryo-susceptible field-resistant varieties described by Batts & Jeater (1958*b*) were winter. Thus it would be necessary to vernalize the seed for at least 6 weeks and sow it in the field at the end of April in order to get full details of any seedling reaction as well as data on the mature plants.

Purvis & Hatcher (1959) observed a tendency for the coleoptile and first few leaves of rye plants from vernalized seed to be shorter than from unvernalized seed. Thus vernalized wheat seeds would probably require shallow sowing to ensure maximum natural emergence through the coleoptile.

Vernalization is effective at a seed moisture content of not less than 50% of its dry weight. The restricted water content checks coleoptile and plumule extension and the plumular bud remains 1–2 mm. from the scutellum. It was therefore decided to combine the obligatory vernalization with an attempt to induce scutellar infection to pass to the plumular bud. Thus, an extended period of vernalization, 10 weeks, at restricted moisture content was used. The mycelium had only to grow approx. 2 mm. during this time to infect the plumular bud.

MATERIALS AND METHODS

The seed used for these experiments was mainly of embryo-susceptible field-resistant combinations but also included intermediate types (Batts & Jeater, 1958*b*). As a control some seed of susceptible combinations was used. Most of the seed had been inoculated by the partial vacuum method in June 1958.

The method of vernalization at restricted water content was similar to that used by Purvis & Gregory (1952). The vernalization dishes were assembled and sterilized at 100° C. for 2½ hr. followed by slow cooling to ensure a level surface of wax. Sterilized filter-papers were slipped into each dish when cool. The seed was surface-sterilized for 5 min. with a 1:1000 mercuric chloride solution and washed well with several changes of sterile distilled water. After soaking in water for 4–5 hr. and the removal of excess water with sterile muslin, the seed was transferred to the dishes. These were then made up with sterile distilled water to the required weight, calculated to increase seed moisture content to 55% of its dry weight, and the seeds were germinated at 15° C. for 24 hr. The dishes were then reweighed, any loss adjusted, and transferred, wrapped in polythene sheeting, to the refrigerator at 4° C. ± 1° C. Each dish was subsequently reweighed each week and the seed maintained at a water content of 50% of its dry weight.

After vernalization the seed was sown on 30 April 1959 in shallow 6 ft. drills, the seeds being spaced approximately 1 in. apart. When the number of emerged seedlings was counted, the soil between each seedling was carefully pricked over to uncover any germinated seeds which had failed to emerge because of a stone or other impediment.

The unvernalized controls were sown in 6 ft. rows on 16 December 1958. The average soil temperature during the remainder of December was 6° C. During the

winter, embryo tests were made on unvernalized samples, some of which were grown in the greenhouse at approximately 65° F.

EXPERIMENTAL

Following vernalization a number of the combinations showed no significant difference in the varietal reaction to loose-smut infection (Table 1), and the amount of mycelium in the embryos varied from very slight to slight. There was no evidence in samples of vernalized seed that the extent of mycelium within the embryo had significantly increased during vernalization. The average emergence was 92%.

Table 1. *Combinations in which vernalization did not affect the normal interaction of host and parasite, and in which no seedling abnormality was observed*

Race C1	Race C2	Race C3
Agror*	Agror†	Agror*
Marne Desprez*	Alter†	Mado*
Minister*	Benign†	Marne Desprez*
Rollo†	Tavero†	Redman*
	9/3/1†	Rollo†

* Embryo-susceptible field-resistant.
† Susceptible.

However, in other combinations, following vernalization a varying number of seedlings exhibited the symptoms of the incompatibility reaction. Such seedlings had stunted growth, and dark green leaves with chlorotic streaks (Pl., fig. 1). Some later produced uninfected tillers.

At first sight this suggested that the long period of vernalization had induced the incompatibility reaction, when normally there was no adverse effect of the pathogen on the seedling stage. Anatomical investigation revealed crown infection, and so it seemed that mycelium had grown through to the plumular bud from the scutellum where, according to Batts & Jeater (1958*b*) and Popp (1959), it was normally confined. Before induction of the incompatibility reaction by vernalization could be claimed, it was necessary to re-investigate the distribution of mycelium in unvernalized seed of these embryo-susceptible field-resistant combinations.

THE REACTION OF INDIVIDUAL VARIETIES

A number of varieties infected with race C2 produced a certain proportion of seedlings clearly showing incompatibility symptoms, and brief details of some of these combinations are summarized in Table 2.

Further investigations were made on unvernalized seed of Aubers and Ideal Bataille. Anatomical investigation of forty-eight Aubers seeds, germinated for 2 days, showed thirty-five embryos with scutellar infection. In only five of these had the pathogen penetrated the plumular bud. A further forty seeds were grown for 3 weeks in the greenhouse, by which time most of the plants were in the 4-leaf stage, and ear initiation had begun. Twenty-three seedlings had scutellar infection, and two of these showed symptoms of epicotyl infection.

Eight Ideal Bataille seedlings showing moderately severe symptoms and having infected epicotyls were observed among 216 seedlings grown to the 2-leaf stage in the greenhouse. Thus in Aubers and Ideal Bataille it is not unusual for a few embryos, having scutella infected with race C2, also to have infected plumular buds, resulting in the incompatibility reaction when the seeds are grown. This is independent of and unaffected by vernalization. It is probable that this principle also applies to the other varieties in Table 2.

After 19 days the normal vernalized seedlings had produced four leaves, while after 26 days five leaves had emerged. Ears emerged June/July.

Table 2. *A summary of the occurrence of seedlings showing incompatibility symptoms, after vernalization of certain wheat varieties infected with loose smut race C2*

	Embryos		Unvernalized autumn-sown seed.	Vernalized spring-sown seed			
					Plants		
Variety	Infection (%)*	Infection intensity†	Total plants‡	% emergence	Total	Smutted	With incompatibility symptoms§
Alfy	64	M.–H.	264	89	313	0	11 (6%)
Atson	28	V.S.	255	95	242	0	12 (18%)
Aubers	40	S.–H.	171	93	335	4	7 (5%)
Ideal Bataille	28	S.–M.	90	96	360	0	10 (10%)
Marne Desprez	33	V.S.	62	96	98	0	10 (31%)
Mesnil	5	H.	45	87	76	0	1 (26%)
Prima	60	S.–H.	123	94	218	0	13 (6%)

* 15–25 embryos tested.
† Very slight, slight, moderate, or heavy embryo infection (Mantle, 1961*b*).
‡ None smutted. No incompatibility symptoms observed.
§ Percentage in brackets indicates the proportion of the total number of infected embryos which produced seedlings with incompatibility symptoms.

Abondance Lepeuple infected with race C2

In this combination scutellar infection often extended into the plumular bud. The extent of penetration was directly associated with the severity of incompatibility symptoms. No smutted ears were recorded. The results, following vernalization, are summarized in Table 3.

In the seedlings showing incompatibility symptoms and dug up 9, 19, 22, 26, 35 and 40 days after sowing, mycelium had reached the apical growing point. In twelve of the thirty-one plants lifted 19–35 days after sowing, uninfected tillers were developing in the coleoptile or first leaf axil.

Thirteen seedlings from thirty-six unvernalized seeds, grown for 12 days in the greenhouse during August 1959, showed distinct incompatibility symptoms. The leaves were shorter than normal, darker green, and displayed chlorotic stripes. The stunted chlorotic seedlings could be divided into two groups. Those with infected apical growing points (Pl., fig. 2) were most severely stunted, having only three leaves above the apex, indicating that no leaves had been initiated since germination. In seedlings with only partial infection of the plumular bud there had been some apical activity (there were 4–5 leaves above this meristem) and the fungus had not

penetrated the crown further than the second node. Infection confined to the scutellum was only associated with a slight restrictive effect on early growth, with no chlorosis.

Where infection was confined to the scutellum there was a recovery, which was almost complete after 12 days, from a slight early inhibition. The seedlings with partial crown infection recovered slightly, while those with infected apical growing points almost ceased growth.

Tests made on batches of unvernalized seed (Table 4), which had been inoculated in 1956, 1958 and 1959, confirmed that in the batch inoculated in 1958 a substantial proportion of infected embryos had mycelium within the plumular bud which, on germination, produced stunted chlorotic seedlings, clearly reacting abnormally to the smut infection. Other seed, inoculated similarly in 1956 and 1959, also showed these reactions.

Table 3. *Summary of reactions of three wheat varieties to infection by loose smut, following 10 weeks' vernalization*

			Plants from vernalized seed					Unvernalized autumn-sown controls	
Variety	Loose smut race	Embryo test: % infection and intensity*	Total no.	% emerged	No. smutted	No. with incompatibility symptoms	% infected embryos producing abnormal seedlings	No. of plants	% smutted
Abondance Lepeuple	C2	36 S.–M.	309	89	0	63	57	68	0
Prima	C1	40 S.–M.	88	92	0	32	91	134	0
Prima	C3	56 S.–H.	115	82	16	16	25	61	20
Dominator	C2	44 M.–H.	114	89	17	0	0	186	0

* Slight, moderate, or heavy embryo infection based on the mycelial distribution in the scutellum.

Prima infected with race C1

In this combination scutellar infection usually extended into the plumular bud and resulted in a typical incompatibility reaction (Table 3). Where the mycelium reached the apical growing point, healthy tillers arose from the coleoptile bud in four plants out of twenty-five. In plants where the mycelium reached the first or second node of the crown, no healthy tillers arose. No smutted ears were recorded in this combination.

Thirty-four plants from unvernalized seeds were grown for 14 days in the greenhouse during August 1959 and examined to determine whether the high incidence of the incompatibility reaction, observed after vernalization, was inherent in the seed or induced by vernalization.

Three distinct groups of seedlings (Table 5), based on the fungal distribution within the host, were identified in this combination. The distinction was evident from leaf, third internode, and rhizome lengths, but whereas apical growing-point infection was consistently correlated with an 81–86% growth reduction on all structures measured, a partial crown infection resulted in almost complete compensation for reduced third internode length by premature extension of the fourth internode (included as part of

the rhizome). Infection confined to the scutellum had no effect on rhizome development but significantly reduced the total leaf length. Three of the plants with severe incompatibility symptoms had an uninfected tiller developing from the coleoptile or first leaf axillary bud.

Table 4. *A classification of unvernalized Abondance Lepeuple seedlings showing incompatibility symptoms associated with infection by loose smut race C 2, from seed inoculated in different years*

		No. of seeds or seedlings				
Year inoculated	Growth	Sown	With scutellar infection	With plumular bud or crown infection	With apical growing-point infection	Showing incompatibility symptoms
1956	To 2-leaf stage in greenhouse	18	3	3	2	3
1958	Germinated 2 days at room temp.	37	14	12	5	Not determined
1959	Germinated 3 days at 5° C. to break dormancy. Grown 10 days in greenhouse	9	6	4	2	4

Table 5. *The summarized effect of the extent of loose smut infection on internode and leaf lengths of unvernalized Prima seedlings, inoculated with race C 1*

		No. of leaves		Mean length (cm.)		
No. of plants	Infection	Above apical growing point	Emerged	Total leaves	Third internode	Rhizome
12	Uninfected	7	3	45·7	0·7	2·1
6	Scutellum only	7	3	35·6	0·7	2·0
3	Partial crown infection. Slight incompatibility symptoms	5	3	17·8	0·3	1·8
13	Apical growing point infected. Severe incompatibility symptoms	3–4	1–3	6·9	0·1	0·4

For comparison, sixteen unvernalized seeds from an ear inoculated in 1959 were grown to the 2-leaf stage and investigated. The three distinct infection groups were again observed showing that this variation occurred even among the progeny of a single ear.

In Prima inoculated with race C 1 there was therefore an inherent tendency for a high percentage of unvernalized seeds with scutellar infection to produce stunted chlorotic seedlings with infected crowns.

Prima infected with race C 3

In this combination, loose smut infection of the plumular bud of the embryo resulted either in the normal susceptible reaction or in the production of the symptoms characteristic of the incompatible reaction (Table 3).

There was a considerable variation in the extent of the stunting of the chlorotic seedlings observed following vernalization, which was not associated with a similar variation in crown infection. After 19 days' growth there were 2–4 leaves emerged and 4–6 leaves above an apical growing point in which the mycelium was present or adjacent. Most of these seedlings had uninfected tillers developing from the bud in the axil of the coleoptile or first leaf.

Although the vernalized seed produced a mixture of plants in which plumular bud infection resulted either in the normal production of smutted ears or an incompatibility reaction in the seedling stage, there was some indication that only a single type of host/parasite interaction was shown by the progeny of each parental ear. Some of the vernalized seed originated from two separate inoculated ears, and after vernalization the progeny from one ear produced smutted plants only, while the other produced stunted chlorotic plants only.

Nineteen unvernalized seeds were grown for 35 days in the greenhouse. One seedling showed clear symptoms: the apical growing point was infected and an uninfected tiller was developing from the coleoptile bud. The remaining seedlings had produced six normal leaves (this was a winter variety), and in ten plants the growing-point region was infected.

For comparison with the 1958 inoculation, twenty-one seeds of Prima inoculated with race C3 in 1959 were germinated for 3 days at 5° C. to break dormancy and sown in the greenhouse. One seedling, infected in the apical growing point, was severely stunted and chlorotic. The normal seedlings were dug up after 16 days. Seventeen plants had scutellar infection and mycelium was found in nine apical growing points. The remaining eight infected seedlings were repotted and grown to maturity under supplementary lighting, and five plants produced smutted ears.

There was thus no significant effect of vernalization on this host/parasite interaction but there was a consistent inherent tendency for plumular bud infection to have an extremely variable effect on the host.

Dominator infected with race C2

In this combination scutellar infection often penetrated the plumular bud. According to the conditions of maturation of the seed so the subsequent plant either showed an incompatibility reaction to infection, or produced smutted ears if growing conditions were suitable.

Seed inoculated in 1958 and matured during the wet cool summer was used in a series of experiments summarized in Tables 3 and 6. Ten ears were threshed separately and the remaining seed was bulked.

Table 6 shows that, although only about 20% of the embryos with infected plumular buds actually had mycelium in the apical growing point, by the 2-leaf stage most of the partially infected plumular buds had developed into completely infected crowns and with continued host growth the pathogen eventually sporulated in the ear.

Unvernalized seeds were germinated for 11 days in the greenhouse. Nineteen seedlings with infected scutella were field-planted in early November 1959 and 74% of the plants produced smutted ears. Only 47% of the infected seedlings from vernalized seeds, grown in the greenhouse for 11 days and planted in early January 1960 in pots

exposed to ambient conditions on the college roof, produced smutted ears; while only 10% smut occurred in a similar vernalized sample sown directly in the field in early April 1960. This emphasizes the importance of early growth at a fairly high temperature to encourage the partial plumular bud infection to invade the crown completely. Subsequent establishment in the field, before soil temperature drops too low, is probably more favourable in allowing the pathogen to keep pace with the growing point than immediate exposure to mid-winter air temperatures.

Table 6. *A summary of the incidence of loose smut infection in the epicotyl of unvernalized inoculated Dominator seedlings at various stages of host development*

Duration of growth	No. of seeds sown	No. of infected scutella*	No. of infected plumular buds or crowns	No. of infected apical growing points
Germinated 2 days at room temperature	20	8	5	1
Grown 10 days to 2-leaf stage in greenhouse	25	7	7	6
Grown 4 weeks to 5-leaf stage in greenhouse	20	8	3	3
To maturity. Scutella removed and tested after 10 days	16	6	Not determined. At least 4	4†

* None of these plants showed incompatibility symptoms.
† Smutted plants.

Table 7. *A summary of the natural incidence of the symptoms of host/parasite incompatibility in unvernalized seed of Dominator inoculated with race C2 in June* 1959

	Seedlings with infected scutella planted in the field			Anatomical investigation				
				No. of seedlings with				
Origin of seed	Total	No. with incompatibility symptoms	Total at maturity*	Total	Infected scutella	Incompatibility symptoms	Crown infection	Apical growing-point infection
5 bulked ears	17	5†	15	40	17	3	3	0
1 ear	8	6‡	7	15	8	4	4	1
1 ear	6	4†	4	16	8	4	4	1
1 ear	7	5‡	6	16	7	3	3	0
1 ear	1	0	1	16	7	5	5	2
Total	39	20	33	103	47	19	19	4

* None smutted.
† 2 seedlings dying } External necrosis proceeding inwards, seedlings dug up in early January 1960. There were 1–2 emerged leaves, 3–4 leaves above the apical growing point, and infection extent varied from 1st leaf insertion to base of apical growing point.
‡ 1 seedling dying }

Two healthy ears from vernalized seed (Table 3) were inoculated with race C2 on 1 July 1959, ripened quickly in the very hot dry weather, and were harvested after 35 days. Four seeds were germinated for 3 days at room temperature and anatomically investigated. All had scutellar and plumular bud infection which, in two embryos, had reached the apical growing point. A further six seeds were grown in the green-

house for 7 days, when the scutella were removed and tested. All the seedlings had scutellar infection and were planted in the field on 4 November 1959. All six seedlings matured to give large plants with smutted ears. No abnormal symptoms were observed.

Healthy ears from unvernalized autumn-sown seed (Table 3) were inoculated with race C2 on 15 June 1959 and were harvested over 2 months later. Four ears were threshed separately, while the seed from five ears was bulked. A sample from each ear and the bulked seed was grown to the 2-leaf stage in the greenhouse and anatomically investigated. Another sample was grown to the 2-leaf stage, the scutella removed, and the seedlings planted in the field on 13 November 1959. The results are summarized in Table 7.

DISCUSSION

The process of vernalization has appeared to have little or no direct effect on the distribution of the fungus within the embryo. However, this technique has proved valuable in the observation of incompatibility symptoms associated with loose-smut infection of some winter varieties, when approximately 90% of the seed, sown in late April, produced seedlings many of which developed normally and came into ear at the usual time. Oort (1944) observed that the abnormal seedlings of 'hypersensitive' combinations rarely emerged in the field, even when sown as late as mid-March.

The observation that vernalization did not affect the incidence of loose smut in normally susceptible combinations (Hanna, 1936; Bever, 1943) was confirmed.

Previous investigators of field resistance associated with embryo infection observed the mycelium confined to the scutellum (Batts & Jeater, 1958*b*) failing to enter the plumular bud (Popp, 1959), and usually confined to the scutellum but occasionally infecting the plumular bud (Ohms & Bever, 1955).

A number of embryo-susceptible field-resistant combinations (Table 1) did not show any seedling reaction, and it may be assumed that in these host/parasite combinations the fungus always failed to penetrate the plumular bud, and consequently the ear was not smutted. Vernalization did not affect this type of resistance, nor did it influence occasional smutting in intermediate types such as Tavero and Benign infected with race C2.

In other combinations, all of which (except Ideal Bataille and Prima infected with races C2 and C1 respectively) had been described as embryo-susceptible field-resistant by Batts & Jeater (1958*b*), seedlings showing incompatibility symptoms were observed following vernalization. In their anatomical investigation of the embryo-susceptible field-resistant reaction of Atson and Aubers infected with race C2, Batts & Jeater did not find any growing-point infection although scutellar infection was often present. In the present work, Atson and Aubers both produced a few abnormal seedlings with infected crowns. Thus in approximately 10% of the infected embryos of these combinations the mycelium was not confined to the scutellum. Where it passed to the plumular bud it induced an incompatibility reaction and, presumably, if the seeds had been sown in early spring, the seedlings would not have emerged.

Prima had been found to resist plumular bud infection by races C2 and C3 (Batts & Jeater, 1958*b*), but here incompatibility associated with crown infection was observed in seedlings infected with race C2. Infection by race C3 resulted in an unusual

reaction, quite different from the observations of Batts & Jeater. Smutted ears were observed from both unvernalized autumn-sown seed and vernalized spring-sown seed. Also, following vernalization, a few plants showed incompatibility symptoms. Further investigation of unvernalized seed from the 1958 inoculation showed this mixture of reactions to be usual for this combination, and this was confirmed by the 1959 inoculation. Inoculation with race C 1, not previously investigated, resulted in high infection, and a large proportion of infected embryos produced abnormal plants. Thus all three Cambridge races are capable of inducing the host reaction.

An entirely new reaction was observed in Dominator infected with race C 2. Vernalization of the seed from five ears resulted in smutted plants from each ear, while another five similarly inoculated ears, which were autumn sown, produced a few more plants but did not produce even one smutted plant. Assuming that there was similar infection in the two groups of five ears, originally grouped randomly from ten inoculated ears, twenty-one smutted plants should have been observed from the autumn sowing. Investigation of unvernalized seed revealed that most of the infected embryos already had infected plumular buds, and a few plants produced smutted ears in the greenhouse. Thus, vernalization was not a prerequisite for the production of smutted ears, but it was a valuable tool whereby the seed could be field-sown in late spring, and grown quickly to maturity. Growth through the winter in the field appeared to be the only factor influencing the possible inability of the fungus to keep pace with the growing point. Abnormal plants were produced by vernalized and unvernalized seed of the 15 June 1959 inoculation, and there was a severe reaction to infection, even though mycelium did not always completely permeate the crown. However, infected seeds from the 1 July inoculation, which were harvested earlier than the 15 June inoculation, all had plumular bud infection, but, when grown, the seedlings did not show abnormal symptoms.

Thus, in Dominator the reaction to race C 2 was extremely variable. Batts & Jeater (1958*b*) had not observed any smut from this combination when winter-sown. This is confirmed although frequent plumular bud infection occurs. In unvernalized seed, symptoms were only observed from the 15 June 1959 inoculation, after which the ears matured in the very hot summer for over 2 months. A long period of maturation at low temperatures and a short period of maturation at high temperatures was sufficient for the fungus to enter the plumular bud, but insufficient for it to induce symptoms. Thus, it was not directly the presence of the fungus in the plumular bud of this variety which induced the unusual reaction. Possibly some metabolic product, produced during the long period of fungal activity within the plumular bud, was responsible. Batts & Jeater (1958*a*) found that, in wheat sown between October and March, the entire plant was laid down by about April. Thus the fungal growth within autumn-sown Dominator had probably been suppressed during the first three months of the year.

Abondance Lepeuple was consistently incompatible with plumular bud infection by race C 2 and the distinct groups of seedling reaction to infection (Mantle, 1961*a*) were also observed here.

Recovery from this reaction by the development of the coleoptile bud, which does not normally produce a tiller in the healthy plant (Percival, 1921), is thus an unusual

feature in some varieties. In incompatible combinations this bud usually escapes invasion during the growth of the pathogen in the plumular bud of the embryo. Extension of the third internode soon isolates the main source of infection, i.e. the plumular bud which has since developed into the crown, and lignification of the hypocotyl region probably prevents mycelium within the scutellum from growing into the coleoptile bud in the early seedling stage. Associated with the inhibition of apical growing-point activity, this axillary bud is stimulated to develop into an uninfected tiller, which produces a healthy ear.

Oort (1944) noticed an effect of temperature during ripening on the degree of 'hypersensitivity', which was presumably related to the extent of fungal distribution within the host, and Dominator illustrates this observation. However, the mechanism which determines the extent of embryo invasion by the fungus is obscure. There is even variation among embryos from one parental ear.

As a result of the present work, in this country at least, the following procedure is recommended for determining the reaction of varieties:

(1) Embryo test. This will establish whether or not the embryos are completely immune to infection.

(2) If embryos are infected, a sample of plumular buds should be dissected from 2-day germinated seed and examined for infection. If there is no plumular bud infection then the variety is field-resistant. Alternatively the method described by Popp (1959) may be used.

(3) If there is plumular bud infection, a further sample of seeds must be grown under greenhouse conditions to observe any seedling reaction to this infection. If there is no reaction the plumular bud infection will probably produce a smutted ear, but in some combinations the mycelium may not keep pace with the growing point and the ears will be healthy. This must be checked by growing to maturity in the field.

Severe seedling reactions to plumular bud infection have a variable economic importance according to the variety/race combination. It is important, however, to identify exactly the type of resistance inherent in parental varieties in breeding programmes for resistance to loose smut. As there are so many different types of resistance it would appear that there must similarly be numerous sets of genetical factors determining the exact form the field-resistance will take. To breed for field-resistance as such is not sufficient. Immunity should be the aim, for this is free from complications. So little is known of the factors which prevent scutellar infection from penetrating the plumular bud, that it is not advisable to rely on the fungus always failing to infect this tissue.

I wish to express my gratitude to Dr O. N. Purvis of the A.R.C. Unit of Plant Physiology and Mr W. Mackenzie, Curator of the Chelsea Physic Garden, for the generous facilities provided for this work, and to Mr A. Horne for taking the photographs.

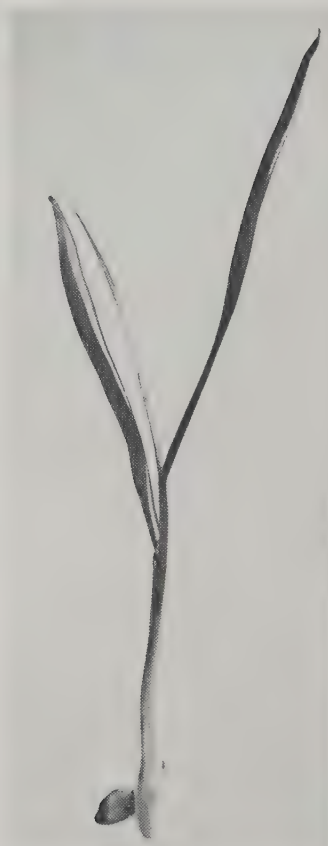


Fig. 1

Fig. 2

REFERENCES

Batts, C. C. V. & Jeater, A. (1958*a*). The development of loose smut (*Ustilago tritici*) in susceptible varieties of wheat, and some observations on field infection. *Trans. Brit. mycol. Soc.* **41**, 115.

Batts, C. C. V. & Jeater, A. (1958*b*). The reaction of wheat varieties to loose smut as determined by embryo, seedling, and adult plant tests. *Ann. appl. Biol.* **46**, 23.

Bever, W. M. (1943). Physiologic specialisation of *Ustilago tritici* and the effect of vernalisation on the incidence of loose smut in artificially inoculated winter wheat. *Rep. 8th E. Wheat Conf. Cincinnati.*

Hanna, W. F. (1936). Effect of vernalization on the incidence of loose smut in wheat. *Sci. Agric.* **16**, 404.

Mantle, P. G. (1961*a*). Further observations on an abnormal reaction of wheat to loose smut. *Trans. Brit. mycol. Soc.* (in the Press).

Mantle, P. G. (1961*b*). Further observations on the reaction of wheat varieties to physiologic races of loose smut. *Ann. appl. Biol.* **49**, 510.

Ohms, R. E. & Bever, W. M. (1955). Types of seedling reaction of Kawvale and Wabash winter wheat to three physiologic races of *Ustilago tritici*. *Phytopathology*, **45**, 513.

Oort, A. J. P. (1944). Hypersensitivity of wheat to loose smut. *Tijdschr. PlZiekt.* **50**, 73.

Percival, J. (1921). *The wheat plant*, 463 pp. London: Duckworth.

Popp, W. (1959). A new approach to the embryo test for predicting loose smut of wheat in adult plants. *Phytopathology*, **49**, 75.

Purvis, O. N. & Gregory, F. G. (1952). Studies in vernalisation. XII. The reversibility by high temperature of the vernalised condition in Petkus winter rye. *Ann. Bot., Lond.*, N.S., **16**, 1.

Purvis, O. N. & Hatcher, E. S. J. (1959). Some morphological responses of cereal seedlings to vernalisation. *J. exp. Bot.* **10**, 277.

EXPLANATION OF PLATE

Fig. 1. Vernalized Aubers seedling showing typical leaf chlorosis due to an incompatible association with loose smut. The infected apical growing point had initiated only four leaves, and uninfected axillary buds were developing within the coleoptile and first leaf axils. (× 1½.)

Fig. 2. Loose smut mycelium permeating the tissues of the third leaf initial and enclosed apical growing point of an Abondance Lepeuple seedling which reacted severely to the presence of the pathogen. (× 100.)

Ann. appl. Biol. (1961), **49**, 704–716
Printed in Great Britain

Potato haulm resistance to *Phytophthora infestans*

III. Lesion distribution and leaf destruction

By D. H. LAPWOOD
Rothamsted Experimental Station, Harpenden, Herts.

(*Received* 23 *March* 1961)

SUMMARY

Reasons were sought for the difference in haulm resistance to potato blight (*Phytophthora infestans* (Mont.) de Bary) of four potato varieties. Up-to-Date produced more foliage than King Edward, Majestic and Arran Viking and a denser canopy which prolonged periods of high relative humidities, and lowered mid-day temperatures by a few degrees. When field conditions favoured infection, the fungus spread in all varieties, but most extensively in King Edward and least in Arran Viking. Infections developed first, mainly in the lower crop canopy, then the middle and finally in the upper canopy, with lesions on leaves and leaflets distributed similarly on all varieties.

The fungus advanced at the same rate in leaf laminae of all the varieties, but not in petioles, which were girdled more rapidly in Up-to-Date and King Edward than in the other two and consequently led to the collapse of more leaves.

Majestic and Arran Viking are more resistant than Up-to-Date and King Edward in the field, because their leaves are infected more slowly early in the blight attack and die more slowly after infection.

INTRODUCTION

When the haulm resistance of four potato varieties to blight (*Phytophthora infestans* (Mont.) de Bary) was assessed during epiphytotics, by comparing the rates at which they became defoliated, their resistance increased in the order King Edward, Up-to-Date, Majestic and Arran Viking (Lapwood, 1961*a*). The fungus spored less on Majestic and Arran Viking than on the other two, but there was no evidence that their leaves were more difficult to infect (Lapwood, 1961*b*). The progress of blight on the four varieties is now analysed in greater detail, from a study of the infection and destruction of individual leaves. Potato haulm is destroyed by the fungus advancing from many initially localized infections: the time taken by the fungus to destroy all the leaves on a plant depends largely on how quickly they all become infected. The rate of initial infection of leaves depends not only upon the host's susceptibility but also on the frequency and duration of weather favouring sporulation of the fungus, and the rate spores are released and dispersed to re-infect the host; the rate of destruction depends on the number and position of the infections on the leaflets of leaves, the rate the fungus advances in the tissues, and on the surface area of leaflets. Some of these factors are considered here.

MATERIALS AND METHODS

Most work was with the varieties Up-to-Date, King Edward, Majestic and Arran Viking, referred to collectively as the standard varieties, but results with the more resistant varieties Ackersegen, Ås and Ontario will also be referred to.

The field observations were made in the potato experiments already described (Lapwood, 1961*a*). Much of the information was obtained from leaves marked by the following method. Five leaves were tagged (with differently coloured polyvinyl chloride wire) on each of ten stems in four plots of each variety. Leaves were selected at five heights equally spaced between the ridge and top of the canopy; the times when five leaflets per leaf (the terminal and the two distal pairs making a total of 1000 leaflets per variety) became infected and destroyed were recorded. In some years the position and number of lesions on leaflets were also recorded but these and other methods are described in the appropriate sections of the text.

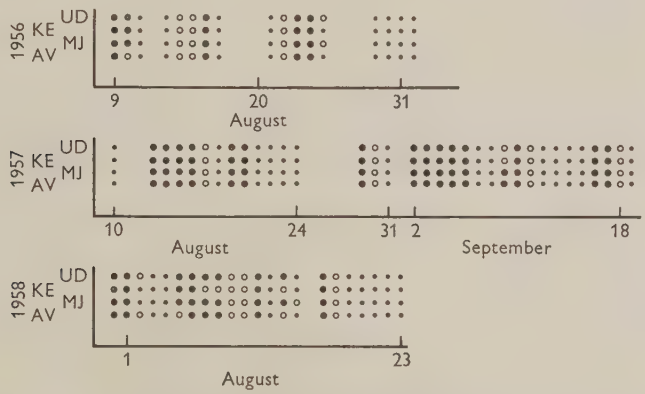


Fig. 1. Frequency of spread of blight in epidemics 1956 to 1958 in plots of the varieties Up-to-Date (UD), King Edward (KE), Majestic (MJ) and Arran Viking (AV). A daily record of new infections with estimates of the numbers of lesions: no symbol, none seen; ●, few (localized); ○, many; ●, abundant (widespread).

RESULTS

(1) *Frequency of periods favourable to spread*

Blight becomes epidemic only in wet seasons, as spread depends on periods of rain and persistent high humidity. The fungus sporulates abundantly only in saturated air (Crosier, 1934) and water must persist as droplets or films on leaves to allow spores time to germinate and penetrate the host.

To investigate the possibility that there were more periods favourable to spread within the canopy in one variety than another, new infections were looked for each day. Laboratory tests, and the exposure of artificially inoculated potted plants in the field within the crop canopy, showed that the fungus had similar incubation time (time

between infection and symptoms showing) on the four varieties. Hence a daily search for new infections should indicate any differences in frequency of spread periods.

Every morning during epidemics crops were inspected for new infections, i.e. lesions of approximately 1 mm. necrosis, which were recorded on a scale: none seen, few, many, abundant (widespread). The daily records for the epidemics 1956 to 1958 (Fig. 1) show that lesions appeared in the four varieties simultaneously and in similar numbers.

Table 1. *Spread of blight around a centrally placed Majestic infector plant in 90 plant plots of each of the standard varieties in* 1956

Variety	No. plants infected	No. of lesions (*a*)*	(*b*)*	Total
Up-to-Date	32	287	94	381
King Edward	42	577	187	764
Majestic	30	227	44	271
Arran Viking	21	162	26	188

* (*a*) Counts on plants in contact with infector plant; (*b*) counts for remainder of plants excluding (*a*).

Table 2. *Estimates of the numbers of lesions involved in the destruction of marked leaflets of the standard varieties in the* 1956 *epidemic, expressed as the average number of lesions found on newly infected leaflets of marked leaves for each observation date*

Variety	August 7	13	16	20	23	27
Up-to-Date	1·6	3·6	8·2	4·0	33·7	13·8
King Edward	1·2	4·0	8·3	2·4	4·9	2·3
Majestic	2·3	3·6	9·8	2·0	45·0	1·9
Arran Viking	1·0	3·2	5·7	2·7	51·0	5·4

(2) *Extent and amount of spread*

In 1956, spread was measured from infected plants placed in the centre of plots of one block of the Latin-square field experiment (Lapwood, 1961 *a*). A potted Majestic plant with seven infected leaflets was exposed in each varietal plot for 5 days in July during a prolonged period of high humidity, before blight had been found in the area, and infections in the surrounding plot were counted during the next few days. Although all the 'infector' plants apparently spored equally, spread to different varieties differed considerably (Table 1) and was most extensive and intensive in King Edward, and least in Arran Viking. In each variety most infections occurred on the six plants touching the infector and the table distinguishes counts on these plants from those of the remainder of the plot.

Similar observations in 1958 and 1959 with replicated plots, and with 'infector' plants of different varieties producing different numbers of spores (see sporing capacity of the standard varieties, Lapwood, 1961 *b*), failed to confirm the earlier result because in both years the period when the infectors were put out coincided with the first extensive spread of the fungus from nearby natural sources of infection.

(3) *Number of lesions*

In 1956, necrotic lesions with a diameter larger than 1 mm. were counted on the tagged leaves in three of the four blocks of the field experiment. Expressing the counts (Table 2) as the average number of lesions found on the newly infected leaflets for each observation occasion, the varieties differ little. The many lesions counted on 23 August indicate the intensity of spread (see Fig. 1) during 2 days of fine rain on 18 and 19 August when spores were produced in abundance and the foliage was wet for long periods. In the King Edward plots 85% of the tagged leaves were already dead and those remaining were near the tops of plants, which accounts for this variety showing fewer new lesions than the others on 23 August.

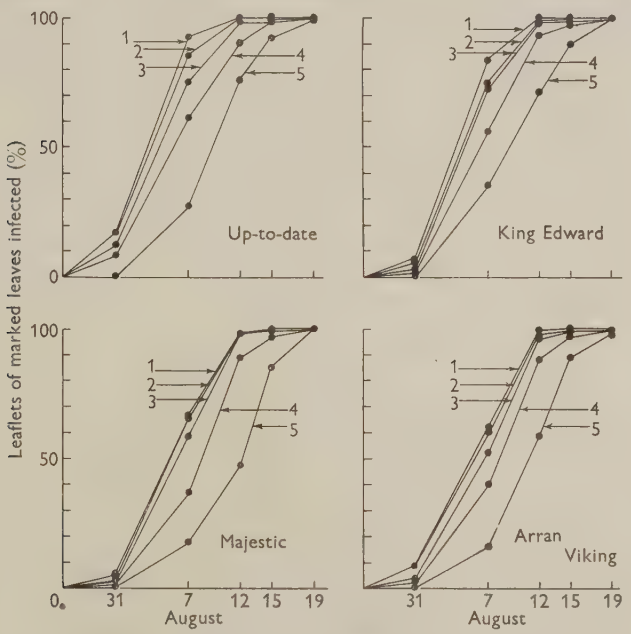


Fig. 2. Effect of blight on the standard varieties, 1958. Rate of infection of leaves marked at five different heights in the crop canopy. The lowermost (1) near the ridge and the uppermost (5) exposed from the crop canopy.

(4) *Position of lesions*

(*a*) *Within the crop canopy*. In the epidemics 1956–58 and for the four standard varieties, the percentage of leaflets infected increased most rapidly first in the lower canopy, then in the middle and finally in the upper canopy (Fig. 2). This sequence probably indicates where environmental conditions most favour infection, but may also reflect spore deposition, for Björling & Sellgren (1955) showed that a sporing source within the crop canopy deposits more spores on middle and lower than upper

leaves. This distribution of lesions can be particularly striking in Up-to-Date, which can appear almost unaffected from outside the crop while the dense canopy is intact, although the whole of the central canopy may have been destroyed. When stems straggle badly after high winds or storms, or when large berries (especially Majestic and Arran Viking) drag down the upper canopy, then these parts are infected as soon as the lower canopy.

(*b*) *On leaves.* On all the standard varieties, the terminal leaflet was most often the first to become infected (in 58% of the marked leaves); the first pair of leaflets was usually infected before the second pair, but opposite leaflets of a pair were equally likely to be infected (41 and 41% for the first pair; 29 and 24% for the second pair).

(*c*) *On leaflets.* The distribution of lesions (1–2 mm. in area) on leaflets was recorded during the early phases of the 1956 epidemic only. The surface of each leaflet was divided into five regions, viz. tip, edge, blade (lamina), midrib and base (insertion of leaf blade and petiole). The area occupied by each of these regions was expressed as a percentage of the whole leaflet (Table 3), so that distribution of lesions on leaflets could be compared with the expected distribution if all regions of the leaflet were

Table 3. *Distribution of lesions on the leaflet surface of the standard varieties in the field. Comparison of the observed distribution (Obs.) with that expected from a chance distribution (Exp.)*

Region	% total area	Up-to-Date		King Edward		Majestic		Arran Viking	
		Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.
Tip	1·0	2	31	3	52	4	37	3	35
Edge	18·0	33	58	48	90	69	89	46	77
Blade	69·0	123	75	179	84	257	197	171	113
Midrib	10·0	18	9	27	29	38	45	25	22
Base	2·0	3	7	5	7	7	8	5	3
No. leaflets		88		135		80		120	
No. lesions		180		262		376		250	

equally likely to be infected. Lesions occurred more often at the tips and edges of leaves of all the varieties than by chance distribution; the leaf blade, although providing the greater proportion of the leaflet area, had fewer lesions than expected (Table 3). The site of infection will depend on where water collects and persists long enough for spores to germinate and penetrate the host, and the position of these sites was indicated by watering plants with whitewash, which on drying was concentrated at leaflet tips and edges. Large, Beer & Patterson (1946) showed that copper deposits were from two to four times as heavy on the tips of leaflets as on the central parts, a distribution which may help spraying to control this disease. These are also places where airborne spores tend to be deposited, for Gregory (1961) showed in wind-tunnel tests that particles are trapped mainly at leaf tips, edges and on the petioles of potato leaves.

(5) *Rate of advance of the fungus in leaf tissue*

Previous work (Lapwood, 1961*b*) suggested that the fungus advanced at a similar rate in the four standard varieties, but rate of advance was further assessed by different methods. At first, three potted plants with fourteen leaves were used and three leaves from consecutive leaf positions 8, 9 and 10 (numbered from the base upwards) were cut from each plant. The terminal and distal pairs of leaflets of each leaf were inoculated on the undersurface, by using filter-paper discs (7 mm. diam.) soaked in inoculum. Two discs were placed on the lamina, one on each side of the midrib of the terminal leaflet; half discs were placed on each side of the midrib of the other smaller leaflets (a total of four inoculations per leaf). A small droplet of inoculum was later introduced under each filter disc to ensure a complete film between the leaf and disc. The leaves were incubated in a saturated atmosphere at 15° C. for 24 hr., after which they

Table 4. *Analyses of variances to show sources of variation in estimates of fungal advance in leaves of seven varieties from the radius (mm.) of chlorotic lesions*

	D.F.	Mean square (variance)	Variance ratio
Between varieties	6	359·78	29·3*
Between plants within a variety	14	61·79	5·0*
Leaf position	2	815·92	66·4*
Residual between leaves	40	12·28	—
Between leaflets	2	5·16	0·47
Residual between leaflets	124	11·05	—

* Variance ratios significant at more than $P = 0{\cdot}01$.

Table 5. *Estimating advance of the fungus in the leaf lamina from the radius (mm.) of chlorotic lesions 5 days from inoculation*

Expt.	Up-to-Date	King Edward	Majestic	Arran Viking	Ackersegen	Ås	Ontario	L.S.D. $P = 0{\cdot}05$
1	6·5	6·4	7·1	6·9	3·5	4·5	4·6	1·97
2	6·0	4·9	6·4	5·2	4·0	3·2	5·2	1·12
3	4·2	4·2	4·9	4·4	3·8	3·1	—	0·80
Mean	5·6	5·2	6·1	5·5	3·8	3·6	—	1·00

were transferred to specimen tubes and kept cool in the light at low humidity. The radii of the chlorotic lesions were measured on two consecutive days using a grid engraved on Perspex (Lapwood, 1961*c*). Tests with the four standard varieties and Ås, Ackersegen and Ontario (Table 4) show not only significant differences between varieties, but also between plants within a variety, and between leaf position on one plant. There is no significant difference between the leaflets inoculated on the same leaf.

The results of three later experiments (Table 5), using more plants and fewer leaves per plant, show no significant differences in the rate the fungus advanced in the standard varieties, but it advanced more slowly in Ackersegen and Ås.

(6) *Leaf areas*

The speed at which a leaflet is destroyed depends on the rate the fungus advances in the tissues, on the number of lesions and the area of the leaflet. In 1957, to compare the composition of the leaf area on the standard varieties, plants were dug from the guard areas of the all-potato experiment (Lapwood, 1961*a*) and sixteen plants of each were taken on three successive weeks. Plants were stripped, their leaves counted, and the number of leaflets estimated from a sub-sample of leaves. Leaf areas were calculated from a further sub-sample of leaflets, by cutting out discs with a punch of known area and then calculating the ratio of area to fresh weight, as described by Watson & Watson (1953). The results from the three sampling occasions analysed statistically as three blocks (Table 6) show obvious differences between varieties. Up-to-Date had significantly greater leaf area and individual leaflets were also larger than on the other varieties. Arran Viking had the same total leaf area as King Edward and Majestic but its leaflets were larger and fewer. King Edward and Majestic have similar leaf area and leaflets but their growth habits differ. Majestic, like Up-to-Date and Arran Viking, is fairly tall, bushy and spreading and described as of the 'British Queen' type (see Whitehead, McIntosh & Findlay, 1945), whereas King Edward is more upright and classified as a different type.

Table 6. *Composition of leaf area of the standard varieties*

(Means per plant from three successive weekly samples, each sample of 16 plants per variety.)

Variety	Leaf area (dcm.^{2})	No. of leaves per plant	No. leaflets per leaf	Leaflet area (cm.^{2})
Up-to-Date	45·3	66	5·7	12·4
King Edward	30·3	89	6·0	5·7
Majestic	30·2	83	5·9	6·2
Arran Viking	29·5	64	4·6	10·4
L.S.D. $P = 0{\cdot}05$	7·40	Not significant	1·03	2·81

Up-to-Date differs from the other varieties in the density of its canopy. To detect gross differences in ecoclimate between the varieties, distance-reading mercury-in-steel wet- and dry-bulb thermographs were installed at ridge height (as described and illustrated by Hirst & Stedman, 1960) before the canopy closed in one plot of each variety. The greatest differences between varieties occurred after the canopy closed, when high relative humidities (about 100%) persisted several hours longer in Up-to-Date than in other varieties, and temperatures at mid-day were often 1 to 2° F. lower.

(7) *Destruction patterns*

The position of a lesion on a leaf or leaflet is important, because it affects the time taken by the fungus to destroy the leaf or leaflet. Records of the affected areas of individual leaflets of tagged leaves often showed that only part of the leaf was destroyed 'directly' by the fungus advancing into leaf tissues; other parts died 'indirectly', from damage to the vascular system. Fig. 3 shows some of the destruction patterns. An infection at a leaflet tip, often on the drooping terminal leaflet of a leaf (Type 1, Fig. 3),

extended until that leaflet was destroyed 'directly'. The fungus then passed into the leaf petiole and as it advanced the pairs of leaflets were destroyed 'indirectly'. A whole leaf is rarely destroyed completely by such enlargement of a single lesion because leaflets usually contract new infections, but it did occur in 1959 when dry weather prevented further infections (see Lapwood, 1961*a*).

Initial infections often occur at the base of the terminal or one of the first (distal) pair of leaflets, because these leaflets overlap and water is trapped and persists between them. Destruction was then usually rapid, as the fungus soon advanced into the

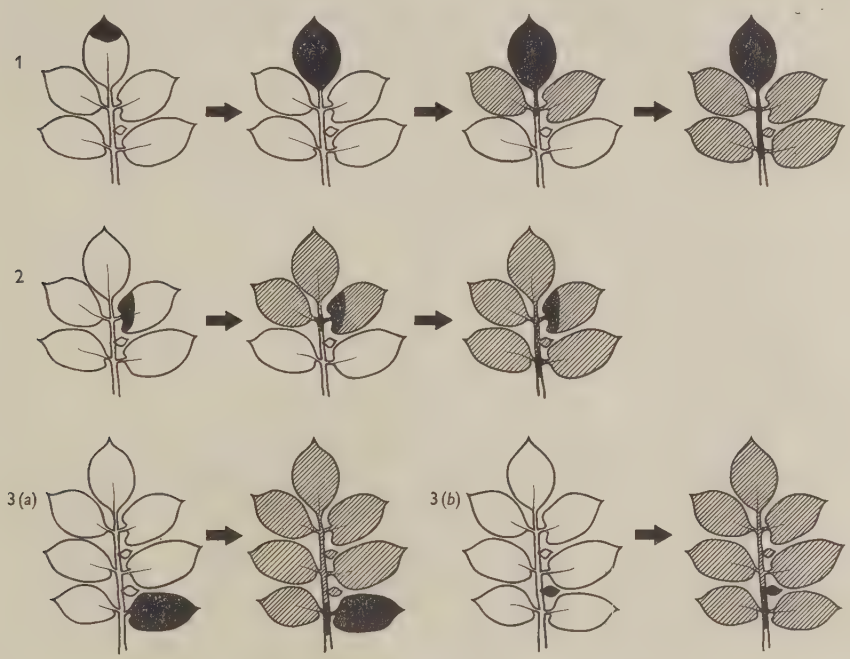


Fig. 3. The effect of the position of a lesion on a leaf and the resulting pattern of destruction by 'direct' and 'indirect' methods (see text). (1) A lesion at a leaflet tip; (2) leaflet base; and (3) in a more proximal position on a leaf (*a*) on a primary, (*b*) a secondary leaflet. Unshaded areas, healthy; shaded areas, destroyed 'indirectly'; black areas, destroyed 'directly'.

leaflet petiole, cutting off the more distal portion of the infected leaflet. On passing into the leaf petiole the pattern was as shown in Type 2 (Fig. 3).

When the petiole was invaded from an infection on a lower primary or secondary leaflet (Type 3*a* and *b*, Fig. 3) the standard varieties differed. Petioles of King Edward and Up-to-Date were rapidly girdled and the leaves collapsed, whereas petioles of Majestic and Arran Viking were attacked along one side and rarely girdled.

Whether leaflets were killed 'directly' or 'indirectly' was recorded from 1956 to 1959. Destruction was assumed to be 'direct' when many coalescing lesions were

found on leaflets, but 'indirect' even when small lesions occurred on leaflets which were turning yellow because of a petiole infection. Table 7 distinguishes between various methods of 'indirect' destruction, (A) resulting from leaflet petiole infection, (B) leaf petiole (Type 3, Fig. 3), and (C) when the fungus passed from leaf infections into and girdled the stem, as in 1959. 'Indirect' destruction was rarest in rapid epidemics, as in 1958, but in all years some leaflets died from leaflet petiole infection (A), usually because of the overlapping of leaflets already mentioned. Varieties differ mainly in the frequency of (B), which reflects differences in petiole resistance between varieties. The few records under category (B) for Majestic and Arran Viking mean only that in these varieties the petioles collapsed so slowly that the leaflets were usually killed first by new infections. This method of presenting results underestimates the resistance of the stems of Arran Viking (C), which were girdled only at the tops of plants, whereas stems of Up-to-Date and King Edward were girdled at any height.

Table 7. *Method of destruction of leaflets of marked leaves within crops in blight epidemics*

(*a*) Destruction of standard varieties 1956 to 1958

	1956			1957*			1958		
		Indirect (%)‡			Indirect (%)			Indirect (%)	
Variety	Direct† (%)	A	B	Direct (%)	A	B	Direct (%)	A	B
Up-to-Date	72	24	4	56	13	30	95	5	0
King Edward	65	16	19	65	10	25	91	6	3
Majestic	71	19	10	75	9	16	92	5	3
Arran Viking	88	11	0·5	85	9	6	99	1	0

(*b*) Destruction of standard varieties, Ås and Ackersegen in 1959

Variety	Other causes (%)	Direct† (%)	Indirect (%)‡ A	B	C
Up-to-Date	18	20	6	45	10
King Edward	13	19	8	47	11
Majestic	15	28	9	40	8
Arran Viking	13	35	8	31	13
Ås	37	21	5	26	10
Ackersegen	67	9	4	18	2

* Results from potato-in-kale experiment only, see Lapwood (1961*a*).

† 'Direct' destruction—leaflet destroyed by the fungus advancing from infections on the leaflet surface.

‡ 'Indirect' destruction (see text and Fig. 3). A, Leaflet destroyed from a leaflet petiole infection; B, leaflet destroyed from a leaf petiole infection; C, leaves destroyed from a stem infection.

In the 1959 field experiment Ås and Ackersegen were much less affected than the other varieties (cf. loss from other causes, Table 7*b*); stems and petioles of Ackersegen were more resistant than the other varieties and many infections were arrested in the leaflets. Rapid independent infections of the leaflet surface can mask 'indirect' destruction. In 1957 when the epidemic developed slowly, only one leaflet was usually infected on a leaf when infection was first noted (Table 8), but in 1958, a particularly rapid and severe epidemic, many leaflets per leaf were infected, although usually fewer leaflets on Arran Viking than on other varieties.

(8) *Petiole resistance*

Petiole resistance was tested on potted plants by placing filter-paper discs soaked in spore suspension at the tip or base of the terminal leaflet, or on a secondary leaflet below two distal pairs of leaflets (see Fig. 3, Type 3). Resistance was estimated by the time the fungus took to destroy 'directly' or 'indirectly' five leaflets, i.e. the terminal and two distal pairs. Table 9, which gives the number of days taken to destroy 50% of the leaflets, shows that King Edward and Up-to-Date were more susceptible than Majestic, and Majestic than Arran Viking, Ås, Ackersegen and Ontario, irrespective of

Table 8. *Numbers of leaflets with lesions, when leaves first recorded as infected on the marked leaves of the standard varieties* (1957 *and* 1958 *epidemics, expressed as a percentage of the total leaves marked*)

No. of leaflets per leaf infected	Up-to-Date (%)	King Edward (%)	Majestic (%)	Arran Viking (%)
1957 1	68	74	76	78
2	21	16	13	12
3	6	8	10	8
4	4	1	0·4	2
5	0	1	0	0
1958 1	16	19	26	31
2	16	13	18	19
3	11	13	15	15
4	21	12	15	19
5	35	42	25	15

Table 9. *Time* (*days*) *for the fungus to destroy* 50% *of leaflets of leaves of potted plants of seven varieties inoculated in different positions, either at the tip or base of the terminal primary leaflet, or on a more proximal secondary leaflet*

Position of inoculation	Up-to-Date	King Edward	Majestic	Arran Viking	Ackersegen	Ås	Ontario
Terminal tip	14	13	14	20	19	>20	>20
Terminal base	10	10	12	15	19	15	15
Secondary leaflet	10	9	15*	Arrested	Arrested	Arrested	>20*

* Some infections arrested.

Table 10. *Rate of advance* (*mm. per day*) *of fungus in midrib tissue of leaves of the standard varieties, assessed by the advance from sporulation or necrosis in advance of sporulation* (*see text*)

(Each figure a 3-day mean from 24 inoculations.)

Variety	Sporulation (mm./day)	Necrotic reaction (mm./day)
Up-to-Date	7·3	7·5
King Edward	6·5	7·9
Majestic	5·1	6·5
Arran Viking	3·7	7·2
L.S.D. $P = 0{\cdot}05$	2·04	Not significant

inoculation position; leaflets died sooner when 'base' and secondary leaflets were inoculated than when the tip was inoculated (except for the secondary leaflet inoculations of Arran Viking, Ackersegen and Ås, where the fungus was arrested and failed to penetrate far into the leaf petiole). This effect with Arran Viking agrees with the field observations and with the estimates of stem and petiole resistance from axillary inoculations (Lapwood, 1961*b*).

The following observations may help to explain the resistance of leaf petioles in Arran Viking. In experiments designed to assess the rate the fungus advances in leaves, leaflets were inoculated at the tips and the fungal advance estimated from the daily extension of sporulation down the leaf midrib, from a reference point near the tip (a spot of coloured waterproof ink). Table 10 shows that the fungus advanced more slowly in Arran Viking than in King Edward or Up-to-Date. However, the sporing zone on Arran Viking was preceded by a continuous necrotic streak in the phloem. In the other varieties necrosis was behind the sporing area, although sometimes and on some leaflets there might be slight necrotic flecks at the surface within and ahead of the sporing area. The necrosis extended along the midrib of Arran Viking at the same rate as the sporing or 'fleck' necrosis extended in other varieties. Cutting leaflets at a distance up to 2 mm. ahead either of the sporing area or of the necrotic streak in Arran Viking did not stop the lesion from developing again, showing that these tissues contained the fungus. Infected tissues of Arran Viking die more rapidly than those of other varieties, and unlike other varieties the fungus seems to advance more readily in the vascular than surface tissues. A delay in fungal spread from the vascular into surrounding tissues may explain the apparent resistance of this variety. Niederhauser, Cervantes & Servin (1954) found that petiole resistance was a feature of resistant clones of *Solanum demissum*.

DISCUSSION

The small differences in blight resistance of haulm between the four varieties selected could be attributed (*a*) to differences in the rate of infection during the early phases of the blight attack, and (*b*) to differences in resistance of invaded leaf petioles, which affected the rate at which infected leaves were destroyed.

Leaves of Majestic and Arran Viking were infected more slowly than Up-to-Date and King Edward at the beginning of the blight attack, probably because lesions on these varieties produced fewer spores (Lapwood, 1961*b*). The length of this 'lag phase' was important as it helped to determine the time taken by different varieties to reach the 50% level of destruction (used as a measure of varietal resistance, see Large, 1945). Once blight became 'epidemic' within plots, the differential effects of inoculum were masked and the four varieties were infected at similar rates (Lapwood, 1961*a*). From Majestic 'infectors' spread was most intensive and extensive in King Edward and least in Arran Viking; therefore, assuming the 'infectors' released similar numbers of spores into the plots of different varieties, other factors besides amount of inoculum must play a part. Van der Zaag (1956) found that spread was greater in plots of Eersteling (Duke of York) than of Eigenheimer, although the fungus produced large numbers of spores on both varieties. Further laboratory tests, using similar

inocula, showed that many more lesions were produced from leaves of Eersteling than Eigenheimer, and he concluded that the latter had a smaller 'chance of infection'. However, in laboratory tests on my standard varieties (Lapwood, 1961*a*), the numbers of lesions did not differ significantly.

In the field, the difference in numbers of lesions on the four varieties (Table 1) may result from factors in addition to host susceptibility and size of inoculum source, for example, the density of the crop canopy, which may affect air movement and the distribution of spores, and the nature of the leaf surface, which may affect the persistence of water and hence the number of infection sites. Thus Arran Viking with its fewer leaflets may expose fewer sites for infection than the other varieties (Table 6).

The effects of age of crop, plant or leaf on susceptibility to blight (Grainger, 1956; Lowings & Acha, 1959; and others) may also be important but they have not been considered in this work. When leaves 8, 9 and 10, detached from pre-flowering plants, were used to estimate (*a*) susceptibility to infection, and (*b*) the rate of fungus advance, there were usually more lesions per unit area on leaf 10 (the youngest) than leaf 8, whereas the fungus advanced more rapidly on leaf 8 than leaf 10—results which agree well with Lowings & Acha (1959). In the field, the effects of crop age and crop ecoclimate on susceptibility to the disease are difficult to separate, because, as the crop develops, the canopy closes and environmental conditions become progressively more favourable to the pathogen (Hirst & Stedman, 1960).

The other main difference between the four varieties was that invasion of the stems and petioles of Majestic and Arran Viking was slower than that of the other two varieties, and this affected the speed at which the fungus destroyed infected leaves. Thus, the average interval between infection and destruction of 50% of the leaflets marked in the different crops was 3·5 and 6·5 days, respectively, for King Edward and Arran Viking (Lapwood, 1961*a*).

The British Mycological Society Key (Anon., 1947), designed to estimate blight in commercial crops, stresses the importance of stem and petiole lesions in destroying King Edward haulm, but at Rothamsted such lesions were less important than leaf lamina infections. In 1956, King Edward haulm from Terrington, a few miles from the Norfolk coast, showed many petiole and stem lesions, often girdling, but few infections on leaflets. If this behaviour is typical of the variety in these commercial areas, it may help to account for the greater differences in resistance reported between King Edward and Majestic growing there. Thus, the differences in field performance of the varieties described here may be of only local value, or perhaps the differences were underestimated because of the proximity of small plots of susceptible and resistant varieties (van der Plank, 1960). However, the differences found by laboratory tests may have wider application and help potato breeders to assess the field merits of the few resistant features that were shown, and thereby help them to assess more readily the type and degree of resistance required to produce successful 'field resistant' varieties.

REFERENCES

ANON. (1947). The measurement of potato blight. *Trans. Brit. mycol. Soc.* **31**, 140.
BJÖRLING, K. & SELLGREN, K. A. (1955). Deposits of sporangia and incidence of infection by *Phytophthora infestans* on upper and lower surfaces of potato leaves. *Acta agric. scand.* **4**, 375.
CROSIER, W. (1934). Studies in the biology of *Phytophthora infestans* (Mont.) de Bary. *Mem. Cornell Univ. agric. Exp. Sta.* no. 155.
GRAINGER, J. (1956). Host nutrition and attack by fungal parasites. *Phytopathology*, **46**, 445.
GREGORY, P. H. (1961). *Microbiology of the atmosphere.* London: Leonard Hill.
HIRST, J. M. & STEDMAN, O. J. (1960). The epidemiology of *Phytophthora infestans*. I. Climate, ecoclimate and the phenology of disease outbreak. *Ann. appl. Biol.* **48**, 471.
LAPWOOD, D. H. (1961*a*). Potato haulm resistance to *Phytophthora infestans*. I. Field assessment of resistance. *Ann. appl. Biol.* **49**, 140.
LAPWOOD, D. H. (1961*b*). Potato haulm resistance to *Phytophthora infestans*. II. Lesion production and sporulation. *Ann. appl. Biol.* **49**, 316.
LAPWOOD, D. H. (1961*c*). Laboratory assessments of the susceptibility of potato haulm to blight (*Phytophthora infestans* (Mont.) de Bary). *Eur. Potato J.* **4**, 117.
LARGE, E. C. (1945). Field trials of copper fungicides for the control of potato blight. I. Foliage protection and yield. *Ann. appl. Biol.* **32**, 319.
LARGE, E. C., BEER, W. J. & PATTERSON, J. B. E. (1946). Field trials of copper fungicides for the control of potato blight. II. Spray retention. *Ann. appl. Biol.* **33**, 54.
LOWINGS, P. H. & ACHA, I. G. (1959). Some factors affecting growth of *Phytophthora infestans* (Mont.) de Bary. I. *P. infestans* on living potato leaves. *Trans. Brit. mycol. Soc.* **42**, 491.
NIEDERHAUSER, J. S., CERVANTES, J. & SERVIN, L. (1954). Late blight in Mexico and its implications. *Phytopathology*, **44**, 406.
PLANK, J. E. VAN DER (1960). Analysis of Epidemics. In *Plant Pathology: an advanced treatise*, Vol. 3. Edited by J. G. Horsfall & A. E. Dimond. New York: Academic Press.
WATSON, D. J. & WATSON, M. A. (1953). Comparative physiological studies of the growth of field crops. III. The effect of infection with beet yellows and beet mosaic viruses on the growth and yield of sugar beet root crop. *Ann. appl. Biol.* **40**, 1.
WHITEHEAD, T., MCINTOSH, T. P. & FINDLAY, W. M. (1945). *The potato in health and disease*, 2nd edition. London: Oliver and Boyd.
ZAAG, D. E. VAN DER (1956). Overwintering en epidemiologie van *Phytophthora infestans*, tevens enige nieuwe bestrijdingsmogelijkheden. *Tijdschr. PlZiekt.* **62**, 89.

Ann. appl. Biol. (1961), **49**, 717–722
Printed in Great Britain

Black-pod disease of cocoa

I. A comparison of isolates of *Phytophthora palmivora* (Butl.) Butl.

By J. A. SPENCE*
Department of Botany, University of Bristol

(*Received* 15 *April* 1961)

SUMMARY

Isolates of *Phytophthora palmivora* from various countries were compared for compatibility in oospore production with standard isolates of the 'rubber' and 'cocoa' groups. It was found that most of the isolates belonged to the 'cocoa' group, one to the 'rubber' group, and three did not form oospores under any circumstances.

The isolates showed different growth rates in wound-inoculated pod tissue. Individual isolates showed different growth rates on pods of two varieties of cocoa.

INTRODUCTION

Black-pod disease of cocoa is caused by *Phytophthora palmivora* (Butl.) Butl. which also attacks rubber, cotton and palms. There is evidence that strains of *P. palmivora* differ in pathogenicity to the various host plants (Ashby, 1929; Tucker, 1931; Orellana & Som, 1959; Turner, 1960).

The commonest symptom of the disease on cocoa is a brown to black rot of the pod. Cocoa pods of all ages may become infected, the lesions produced spreading uniformly from either end of the pod, or forming circular to elliptical discoloured and rotted areas around the middle.

The fungus may also attack the leaves, inducing irregular necrotic lesions which later become blackened, dried up and crumbled. The foliar disease is of little importance in most countries.

Attack of the bark causes brown to black discoloured lesions which may spread in young stems to cause die-back, but remain small on woody stems. The bark disease was formerly of importance but has been countered by the use of varieties with resistant bark. Unfortunately, these varieties are not equally resistant to pod rot.

There are no varieties of cocoa immune to pod rot and few show resistance.

As a preliminary to a study of possible resistance mechanisms the relative pathogenicity of isolates of *P. palmivora* from various cocoa-growing areas to a number of cocoa varieties was examined.

* Present address: c/o Ministry of Agriculture, Lands and Fisheries, Port of Spain, Trinidad, West Indies.

COMPARISON OF A NUMBER OF ISOLATES OF *PHYTOPHTHORA PALMIVORA*

Oospore production

Gadd (1927) and Ashby (1929) divided isolates of *P. palmivora* into 'cocoa' and 'rubber' strains, according to their compatibility with stock strains originally isolated from rubber and cocoa respectively. When a member of one group is grown on suitable media with a member of the other, oospores are formed in a few days.

Turner (1960) assigned West African isolates of *P. palmivora* from cocoa to the cocoa or rubber strains or to a third group which did not produce oospores with either cocoa or rubber types.

In the present study, isolates of *P. palmivora* were obtained from the Commonwealth Mycological Institute, Kew, and as recent isolates from the main cocoa-growing areas.

Carrot agar was the most satisfactory medium for the typing of isolates since it was transparent, encouraged the production of both sporangia and oospores, but induced only limited aerial mycelium, thus permitting ready observation.

The isolates listed in Table 1 were tested for their ability to form oospores with the standard C.M.I. strains WA 8 ('rubber' group) and WB 9 ('cocoa' group) which produced abundant oospores when grown together.

The test plates were inoculated with discs (4 in. diameter) taken from the advancing edge of carrot agar cultures of the relevant isolates.

Table 1. *Formation of oospores by isolates of* Phytophthora palmivora *in mixed culture with standard rubber and cocoa strains (WA 8 and WB 9 respectively)*

Isolate number	Country of origin	Source	Production of oospores with WA 8	Production of oospores with WB 9
GA 1	Ghana	C.M.I. no. 63552	+	–
GB 2	Ghana	C.M.I. no. 74805	+	–
IA 12	Ivory Coast	Cocoa pod	+	–
NA 4	Nigeria	C.M.I. no. 63604	–	–
NB 10	Nigeria	Cocoa pod	–	–
BA 11	British Cameroons	Cocoa pod	–	–
JD 16	Jamaica	Cocoa pod	–	+
JA 13	Jamaica	Cocoa pod	+	–
JB 14	Jamaica	Cocoa pod	+	–
JC 15	Jamaica	Cocoa pod	+	–
JE 17	Jamaica	Cocoa seedling	+	–
TA 3	Trinidad	C.M.I. no. 74798	+	–
CA 5	Costa Rica	Cocoa pod	+	–
CB 6	Costa Rica	Cocoa leaf	+	–
CC 7	Costa Rica	Cocoa bark	+	–

+ indicates abundant production of oospores in 10 days; – indicates no oospores produced after 4 weeks.

No strain produced oospores when paired with itself. Isolate JD 16, from Jamaica, produced oospores with WB 9 but not with WA 8. Thus JD 16 belongs to the 'rubber' group. The other four Jamaica strains (JA 13, JB 14, JC 16 and JE 17) produced oospores with WA 8. Pairings of JD 16 with each of the other four Jamaica isolates produced oospores. Turner (1960) showed that the common Nigerian strain of *P. palmivora* belonged to the 'rubber' group.

NA4 and NB10 from Nigeria and BA11 from British Cameroons failed to form oospores with either of the test strains. Tests described later show that all three produce typical black-pod lesions on cocoa pods.

Growth rate of isolates in wound-inoculated cocoa pods

Pods sent by air from Ghana (West African Amelonado) and Trinidad (Varieties ICS 1 and SCA 6) were inoculated in England by removing a plug of pod tissue, 4 mm. diam. and 3 mm. deep, and replacing it by a plug from the advancing edge of a carrot

Table 2. *Comparison of growth rates of different isolates on pods of one of two varieties of cocoa, with isolate GA 1 as standard in each series*

Variety	Isolate	Average diameter of lesion after 3 days (cm.)	Figures adjusted to standard GA 1 as 100
West African Amelonado (10 wounds per treatment, p. 720)	GA 1	4·1	100
	GB2	3·9	95
	Control	0	0
	GA 1	4·3	100
	TA3	4·5	105
	NA4	3·5***	84
	Control	0	0
	GA 1	4·4	100
	CA5	3·7***	84
	CB6	4·6	105
	Control	0	0
	GA 1	4·0	100
	WA8	0***	0
	NB10	3·6	90
	Control	0	0
	GA 1	4·3	100
	BA11	3·4***	79
	IA12	4·1	95
	Control	0	0
	GA 1	3·9	100
	JA13	4·0	103
	Control	0	0

Variety	Isolate	Average diameter of lesion after 5 days (cm.)	Figures adjusted to standard GA 1 as 100
ICS 1 (9 wounds per treatment, p. 720)	GA 1	4·5	100
	GB2	4·4	98
	NB10	3·8	84
	Control	0	0
	GA 1	4·2	100
	NA4	3·7	88
	BA11	2·1***	50
	Control	0	0
	GA 1	4·0	100
	IA12	4·0	100
	JA13	3·7	93
	Control	0	0

*** Significantly lower than standard isolate GA 1 at 0·1 % level.

agar culture of *P. palmivora*. In the main trial inoculations were made on the pod ridges, as a preliminary experiment had shown no significant difference between results of inoculations on the ridges and in the furrows. The wound was sealed with a drop of a melted wax-'Vaseline' mixture and the pods incubated at 25° C. The diameters of the circular lesions produced at the pod surface were measured in two directions at right angles. By the time that the lesion was visible on the surface, rotting had penetrated through the shell and its subsequent spread in the tissue was at a similar rate to that of the surface lesion.

Discs from agar cultures of selected isolates were placed at three sites on ridges of cocoa pods (except with pods of West African Amelonado, which were inoculated at two sites only). Sterile agar plugs were used for controls. Each pod was inoculated with isolate GA 1 as standard and with two other isolates to be tested. Thus four ridges were treated on each pod. On the smaller West African Amelonado pods, each treatment was replicated on five pods giving a total of ten wounds per treatment. On the cocoa varieties ICS 1 and SCA 6 the limited number of pods available limited replication to three pods (i.e. nine wounds per treatment). The lesions developing on pods inoculated in this way remained distinct from one another for at least 3 days.

Results are shown in Tables 2 and 3.

Table 3. *Comparison of growth rates of selected isolates on pods of varieties of cocoa. Nine wounds per treatment*

Variety	Isolate	Average diameter of lesion after 5 days (cm.)	Significant differences 5 %	1 %	0·1 %
ICS 1	GA 1	4·3			
	TA 3	4·0			
	NA 4	2·2			
	Control	0	0·27	0·38	0·55
SCA 6	GA 1	2·2			
	TA 3	2·0			
	NA 4	2·0			
	Control	0			
ICS 1	GA 1	4·8			
	CA 5	4·5			
	WA 8	0			
	Control	0			
SCA 6	GA 1	3·3	0·92	1·40	2·25
	CA 5	3·0			
	WA 8	0			
	Control	0			

Tables 2 and 3 show that, on the varieties of cocoa used, most of the isolates of *P. palmivora* grew as fast as the standard GA 1, but that a few (i.e. NA 4, NB 10, BA 1 and CA 5) have a significantly slower growth rate. One isolate, WA 8, did not attack any pods into which it was inoculated. Isolates GA 1, TA 3 and CA 5 grew more slowly on pods of variety SCA 6 than on ICS 1, but the slow-growing isolate NA 4 grew at the same rate on both these varieties.

DISCUSSION

Turner (1960) divided West African isolates of *P. palmivora* into three groups—Ghanaian (G), Nigerian (N) and Angolan (A)—on the basis of sporangial dimensions, oospore production and characteristics of the lesion produced on pod tissue.

Turner's G group isolates would be 'cocoa' strains as classified by Gadd (1927) and Ashby (1929), and his N group isolates would be 'rubber' strains. His A group isolates did not produce oospores.

Leonian (1934) showed that when a particular isolate of *Phytophthora* was inoculated into a range of host species the sporangia subsequently produced on these hosts showed variations comparable with those between sporangia produced by different isolates on the same host.

No attempt has been made in the present study to relate differences in the type of lesion produced with strain differences. The same isolate inoculated into different cocoa varieties produced lesions differing from one another in appearance and size as much as did those produced by different isolates on the same host variety. Thus the type of lesion would appear to be a function of the host/parasite relationship rather than a fundamental characteristic of the parasite.

Of the three characters used by Turner only oospore production has been recorded for all the relatively few isolates used in the present study. Most of these would belong to Turner's 'G' group if classified on the basis of oospore production alone. One (JD 16) would belong to his 'N' group and three (NA 4, NB 10 and BA 11) to his 'A' group. NA 4 and NB 10 are from Nigeria and BA 11 from the Cameroons. Turner does not report any 'A' group isolates from these countries. However, he (personal communication) used blocks of infected cocoa-pod tissue for inoculation of the agar plates used for oospore production tests and this may explain the fact that oospores were produced by isolates from Nigeria and the Cameroons in his tests but not in the present study. The blocks of tissue might well have supplied some metabolite essential for oospore production but which these strains were unable to synthesize and which was lacking in the agar medium.

Growth rates of isolates were determined in the present study on pods of West African Amelonado, which is propagated from seed and is thus variable, and on pods of clonal varieties ICS 1 and SCA 6. More than one test isolate were inoculated together with a standard isolate on individual pods and this allowed statistical analysis of both strain differences and pod differences to be made. Significant differences were observed between individual pods in the series of tests with West African Amelonado but not with the clonal varieties.

The differences observed between different isolates on the same host and between the same isolate on different hosts suggest that growth rate on pod tissue is also a function of the host/parasite relationship. Grouping according to oospore production does not necessarily coincide with grouping according to growth rate on pod tissue. Thus CA 5 which is significantly slower growing than the standard GA 1 would be classified in Turner's 'G' group on oospore production, whereas the isolates NA 4, NB 10 and NA 11 (Turner's 'A' or 'N' group) are also significantly slower growing than the standard isolate GA 1 from Ghana which falls into Turner's 'G' group.

A more detailed study has been made of the relation between selected varieties of the host and a limited number of isolates of *P. palmivora*, the results of which are given in the next article.

Since this paper went to press Turner (1961) has published an account of his studies on isolates of *P. palmivora* from non-African countries. Most isolates were of the 'cocoa' type, but the 'rubber' type also occurred.

My sincere thanks are due to Dr L. E. Hawker for helpful advice and criticism. I am indebted to the West African Cocoa Research Institute, Ghana, and the Cocoa Board, Trinidad, for sending cocoa pods by air. Cultures of *P. palmivora* were kindly supplied by the Commonwealth Mycological Institute, Kew, and by plant pathologists in the various cocoa-growing countries. Thanks are also due to Miss G. M. Waterhouse for advice on methods of typing isolates of *P. palmivora*, and to Mr G. M. Clarke of Long Ashton Research Station who carried out the statistical analysis of results. The work forms part of a thesis submitted for the Degree of Ph.D. of the University of Bristol and was made possible by a grant from the Government of Trinidad and Tobago.

REFERENCES

Ashby, S. F. (1929). Strains and taxonomy of *Phytophthora palmivora* Butler (*P. faberi* Maubl.). *Trans. Brit. mycol. Soc.* **14**, 18.

Gadd, C. H. (1927). The relationship between the Phytophthorae associated with the bud-rot disease of palms. *Ann. Bot., Lond.*, **41**, 253.

Leonian, L. H. (1934). Identification of *Phytophthora* species. *Bull. W. Va. Agric. Exp. Sta.* no. 262.

Orellana, R. G. & Som, R. K. (1957). Correlation between low temperature and *Phytophthora* pod rot of cacao in Ceylon. *F.A.O. Plant Protection Bulletin*, **6**, 6.

Tucker, C. M. (1931). Taxonomy of the genus *Phytophthora* de Bary. *Res. Bull. Univ. Mo. Agric. Exp. Sta.* no. 153.

Turner, P. D. (1960). Strains of *Phytophthora palmivora* (Butl.) Butl. from *Theobroma cacao* L. I. Isolates from West Africa. *Trans. Brit. mycol. Soc.* **43**, 665.

Turner, P. D. (1961). Strains of *Phytophthora palmivora* (Butl.) Butl. from *Theobroma cacao* L. II. Isolates from non-African countries. *Trans. Brit. mycol. Soc.* **43**, 665.

Ann. appl. Biol. (1961), **49**, 723–734
With 1 plate
Printed in Great Britain

Black-pod disease of cocoa

II. A study of host–parasite relations

By J. A. SPENCE*
Department of Botany, University of Bristol

(*Received 22 April* 1961)

SUMMARY

Active pectolytic enzyme could not be recovered from cocoa-pod shell tissue parasitized by *Phytophthora palmivora*, but was recovered from autoclaved potato blocks invaded by this parasite. Browned extracts of cocoa bean and pod tissue inactivated pectolytic enzymes of *P. palmivora* obtained from invaded potato blocks. When allowed to brown before exposure to enzyme, discs of cocoa-pod tissue showed increased resistance to maceration by pectolytic enzymes of *P. palmivora*.

Attack of cocoa pods by a weakly virulent isolate was promoted by low oxygen tension and low temperature. Attack by a virulent strain was accelerated at low oxygen tension and retarded at high oxygen tension.

The results suggest that a balance exists during parasitism of cocoa pods by *P. palmivora* between the pectolytic enzyme system of the fungus and the polyphenol oxidase system of the host and that the activity of the polyphenol oxidase system acts as a host resistance mechanism.

INTRODUCTION

Black-pod disease of cocoa (*Theobroma cacao* L.), caused by the fungus *Phytophthora palmivora* (Butl.) Butl., occurs in all cocoa-growing areas. Padwick (1956) estimates crop loss as 50,000 tons per annum, i.e. approximately 10% of world production.

The fungus attacks pods, leaves and bark of the cocoa tree but the commonest and most serious symptom is a brown to black rot of the pod. A partial control of the disease is achieved by field sanitation and fungicidal sprays, but the degree of control varies in different areas. No entirely immune varieties of cocoa are known. Although several varieties are resistant to attack on the bark, only a few show any resistance to rotting of the pods. Knowledge of the mechanism of such resistance is an essential preliminary to any attempt to control the disease by plant breeding.

Surface contamination of the pod by sporangia conveyed from diseased pods by wind or rain readily occurs in the field. Under suitable weather conditions germ tubes produced directly from the sporangia or from zoospores penetrate the pod via the stomata or directly through the cuticle (Rorer, 1910). The fungus then spreads through the shell causing a dark, firm rot, the whole pod finally becoming blackened.

* Present address: c/o Ministry of Agriculture, Lands and Fisheries, Port of Spain, Trinidad, West Indies.

Resistance might occur either during or after penetration. The present study is concerned with post-penetration resistance.

De Bary (1885) and Marshall Ward (1888) showed that rotting of plant tissues by pathogenic organisms involved the dissolution by enzyme action of the pectic layers of the middle lamella of the cell walls. Brown (1915) extracted pectolytic enzymes from germ tubes of *Botrytis cinerea* and showed that both crude and partially purified enzymes reproduced the macerating effect in various plant tissues. The work of Brown and his students (Brown, 1955) showed that host resistance was correlated with the reaction between the host tissues and the pectolytic enzymes produced by the pathogen. In the present paper the writer has applied Brown's pectolytic enzyme theory of parasitism to a study of the host–parasite relation in black-pod disease of cocoa.

MATERIAL AND METHODS

The experimental work was carried out partly in the Department of Botany, University of Bristol, England, and partly in the Department of Botany, University College of the West Indies, Jamaica. While Jamaica could provide a supply of cocoa pods, it had the disadvantage that quarantine regulations limited the experimental material to those varieties of cocoa in cultivation in the island and to isolates of *P. palmivora* obtained from such material.

(1) *Isolates of* Phytophthora palmivora. Isolates were obtained from the Commonwealth Mycological Institute (C.M.I.), Kew, England, and from plant pathologists in West Africa, the West Indies and Costa Rica (Spence, 1961). Three isolates were selected for use in the present study: GA 1, a virulent cocoa strain from Ghana; JB 14, a virulent cocoa strain from Jamaica; and WA 8, a rubber strain avirulent on cocoa, non-sporangial, obtained from C.M.I., country of origin unknown.

(2) *Varieties of cocoa.* For the work in England, pods of the clonal varieties IMC 67, SCA 6 and ICS 1 were obtained from Trinidad by air. These reached Bristol 48 hr. after gathering and arrived undamaged by bruising or frost.

The Trinidad varieties used in Bristol were not available in Jamaica and could not be imported. The Jamaican variety ICS 60 was used in all experiments there.

Wound inoculation experiments (Spence, 1961) showed that the variety SCA 6 was partially resistant to *P. palmivora*.

(3) *Estimation of pectolytic activity.* Brown's (1915) method was adopted. Discs 0·5 mm. thick were cut from a cylinder of tissue extracted from a potato tuber with a no. 3 cork borer (diameter 8 mm.). After exhaustion in water under vacuum they were immersed in 1 ml. of test solution to which was added 1 ml. of McIlvaine's phosphate-citrate buffer pH 5·0 to give a test solution of pH comparable with that of the cocoa pod tissue. The maceration time in minutes (average of four discs) was taken as the time required for the discs to lose coherence under a slight pull from opposite sides with forceps, readings being taken at 10 min. intervals. No experiment involving maceration of potato discs was continued for more than 6 hr.

A method for measuring polygalacturonase, a specific pectolytic enzyme, was described by Dingle, Reid & Solomons (1953). This was also used in the present study and gave results comparable to those obtained by Brown's method.

The assay of specific pectolytic enzymes, such as polygalacturonase, may be of some value in investigations on the process of rotting, but direct measurement of loss of coherence of plant tissue is still the most satisfactory basis for the estimation of macerating activity of enzymes produced by parasites of the rot-producing type.

RESULTS

(1) *Production of pectolytic enzymes by* Phytophthora palmivora

Attempts were made to demonstrate pectolytic enzyme activity in rotted pod shell tissue. The presence of large quantities of mucilage in this tissue together with the firm nature of the rot made it impossible to extract juice by the usual mechanical methods. An aqueous extract of ground tissue was prepared by grinding 5 g. rotted pod shell tissue with 20 ml. distilled water. The vacuum filtered filtrate was tested for macerating enzyme.

Menon (1934) found enzymes capable of macerating plant tissue in extracts of potato blocks parasitized by *Phytophthora erythroseptica*. Potato blocks approximately 6 cm. long cut with a no. 12 borer (diameter 18 mm.) were placed in boiling tubes on moistened cotton-wool, autoclaved and inoculated with a plug from a carrot agar culture of *P. palmivora*. After 3 weeks, when mycelial growth had penetrated throughout the blocks, they were squeezed in muslin to give about 5 ml. of crude extract.

P. palmivora was also grown on potato extract and on various synthetic media as used by Ashour (1954) and Gupta (1956) for the production of pectolytic enzymes by *Pythium debaryanum*, and by Cole (1956) for the production of such enzymes by *Sclerotinia fructigena*.

No pectolytic activity could be demonstrated in extracts of parasitized cocoa-pod shell tissue or in liquid media in which *P. palmivora* had been grown. An active enzyme was present, however, in invaded autoclaved potato blocks (Table 1) and these were used as source of enzyme in later experiments.

The use of distilled water in extraction of pod shell tissue would have diluted an enzyme present. A comparable extract from potato blocks was extracted from 5 g. of invaded potato tissue with 20 ml. distilled water. Table 2 shows that this amount of dilution of potato block extract did not remove measurable enzyme activity.

Tests for polygalacturonase enzyme activity carried out by the agar plate method (p. 724) gave similar results.

(2) *Deactivation of fungal pectolytic enzymes by host extracts*

The lack of demonstrable pectolytic activity in cocoa-pod tissue rotted by *P. palmivora* suggests a comparison with the 'brown rot' produced by *Sclerotinia fructigena* in apple fruit, where J. S. Cole (1956) found little or no pectolytic activity. This worker and M. Cole (1958) showed that oxidized apple juice deactivated pectolytic enzymes produced by *S. fructigena* in culture media and considered that this deactivation explained the failure to demonstrate pectolytic enzymes in rotted apple tissue. Byrde (1957) suggested that the rapid browning of high tannin varieties of apple might be related to their relatively high resistance to attack by *S. fructigena*.

Extracts of cocoa bean or cocoa-pod shell tissue were made in distilled water, in which rapid browning took place, or in 0·1 % aqueous solution of sodium dithionite, in which browning was prevented. Beans had the advantage that they contained less

Table 1. *Pectolytic enzyme activity of extracts of parasitized pod shell tissue and invaded autoclaved potato tissue*

Source of enzyme	Maceration of potato discs (time in min.). Isolate of *P. palmivora*	
	GA 1	WA 8
Parasitized pod shell extract	∞	–
Invaded potato block extract	113	98
Control (potato block extract)	∞	∞

∞ Denotes that when the test was terminated after 6 hr. maceration of discs had not taken place.
– No test.

Table 2. *Pectolytic enzyme activity of extracts of parasitized pod shell tissue, invaded potato tissue and diluted extract of invaded potato tissue. Isolate GA 1*

	Maceration of potato discs (time in min.)
Parasitized pod tissue extracted with 20 ml. distilled water	∞
Invaded potato block extracted with 20 ml. distilled water	170
Invaded potato block undiluted sap extract	113
Control (potato block sap extract)	∞

∞ Denotes that when the test was terminated after 6 hr. maceration of discs had not taken place.

mucilage than did the cocoa-pod shell tissue. Griffiths (1958) showed that two of the three main polyphenols causing browning in the bean are present in shell tissue. Oxidized (browned) and unoxidized extracts were tested with crude pectolytic enzyme preparation from potato blocks invaded by selected isolates of *P. palmivora*, both alone and with the addition of gelatin, shown by Byrde (1957) to remove the inactivating effect of browned apple juice on pectolytic enzymes. Table 3 shows the results of a typical experiment with bean extract. Other experiments gave comparable results with either bean or shell extract. The shell extract showed slightly less inactivating effect than did the bean extract, the maceration time for enzyme produced by isolate GA 1 and treated with unoxidized and oxidized bean extract of var. SCA6 being 165 min. and infinity respectively; that for shell extract being 125 and 263 min. Comparable extracts of var. ICS 1 gave maceration times of 170 min., infinity, 120 and 285 min.

These results indicate that the pectolytic enzymes of *P. palmivora* are inactivated by aqueous extracts of shell and bean which have been allowed to brown, but if browning is prevented, the inactivation is considerably reduced or eliminated. The deactivating power of browned extracts is removed by gelatin which, according to Byrde (1957), indicates that inactivation is due to a tannin-like substance of high

molecular weight, presumably produced by the oxidation of polyphenols, the products of which reaction polymerize to give the brown compounds.

The effect of pectolytic enzymes of *P. palmivora* in macerating discs of cocoa-pod shell tissue was also investigated. Two varieties of cocoa were used: ICS 1—susceptible in the field; and SCA6—resistant in laboratory tests (Holliday, 1954). Tissue discs, diameter 6 mm. and 0·25 mm. thick, were placed in a 0·1 % sodium dithionite solution either immediately after cutting or after being allowed to brown for stated intervals. A control set was placed in distilled water. Pectolytic enzyme of *P. palmivora* from autoclaved potato blocks was buffered at pH 5·0 as previously described and added in equal quantity to the solutions containing the discs, and the time for maceration of the discs was recorded.

Table 3. *Effect of bean extracts of two varieties of cocoa and of bean extract after treatment with gelatin, on activity of pectolytic enzymes of* Phytophthora palmivora

Additions to pectolytic enzyme extract	Cocoa variety	Maceration time (in min.). Isolates		Polygalacturonase activity (p. 724) (diameter of ring in cm.). Isolates	
		GA 1	WA 8	GA 1	WA 8
Oxidized bean extract	SCA 6	∞	∞	0	0
	ICS 1	∞	∞	0	0
Unoxidized bean extract (treated sodium dithionite)	SCA 6	118	125	1·4	1·7
	ICS 1	120	145	1·4	1·8
Oxidized bean extract + gelatin	SCA 6	105	103	1·4	1·9
	ICS 1	103	108	1·6	1·8
Gelatin	—	108	98	1·8	1·8
Sodium dithionite	—	105	118	1·6	1·8
Distilled water	—	98	108	1·6	1·8

∞ Denotes no maceration of discs at end of experiment after 6 hr.

Table 4. *Effect of pectolytic enzyme of* Phytophthora palmivora *on partially and completely oxidized discs of cocoa shell tissue* (*maceration time in minutes*)

Oxidation time (i.e. time before addition of sodium dithionite)	Variety	
	ICS 1	SCA 6
1 min.	90	180
5 min.	113	∞
15 min.	303	∞
Complete oxidation	337	∞

∞ Denotes that when the test was terminated after 6 hr. maceration of discs had not taken place.

Table 4 shows the effect of pectolytic enzymes of *P. palmivora* in macerating cocoa-pod tissue and the effect of degree of browning of the tissue on resistance to maceration. Discs of var. SCA6 browned more rapidly than did discs of var. ICS 1. The trial was repeated with similar results.

These results show that resistance to maceration of discs of cocoa shell tissue increases with the extent of browning of the tissue. Variety SCA6, reported to show resistance (Holliday, 1954), both browned more rapidly and showed greater resistance to maceration.

The oxidized extracts of cocoa have a much greater effect on deactivating pectolytic enzymes of *P. palmivora* than has apple juice on pectolytic enzymes of *Sclerotinia fructigena* (Byrde, 1960; Cole, 1956). It is therefore not surprising that an active pectolytic enzyme could not be demonstrated in cocoa-pod shell tissue rotted by *P. palmivora*.

Griffiths (1958) has shown by chromatographic methods that cocoa-pod shell tissue contains polyphenols and polyphenol oxidase enzymes which together give rise to browning on mechanical injury of the pod. It is presumably this reaction which occurs when tissues are invaded by *P. palmivora*.

Using Griffiths's chromatographic methods the writer demonstrated the presence in cocoa-pod tissue of the major polyphenols epicatechin, leucocyanidins L_1 and L_2 and an unidentified polyphenol. No *qualitative* difference was observed in the polyphenols present in the resistant variety SCA6 and the susceptible variety ICS1. The possibility exists, however, that *quantitative* differences in polyphenolic content, or differences in the oxidizing enzymes of the two varieties could give rise to different rates of browning of tissue.

(3) *Effect of oxygen tension and temperature on host–parasite relations*

Since the effect of oxidized host extracts is to inhibit activity of pectolytic enzymes of *P. palmivora*, it is possible that during rotting a balance exists between pectolytic enzyme production by the pathogen and inactivation by the host. If this balance could be disturbed in favour of the pathogen, it might be possible to induce an avirulent strain, such as WA8, to attack pods. Factors such as oxygen tension and temperature, which influence the production of the brown tannin-like substances by the pod, might influence attack by the fungus.

(*a*) *Effect of temperature*

Pods were inoculated by the agar-culture plug technique (Spence, 1961) and incubated at 15°, 25° and 30° C. at high humidity.

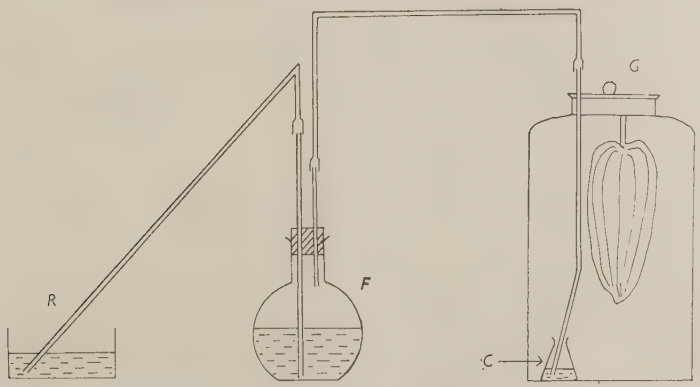
Pods of variety ICS60 were each inoculated in six places, with a weakly virulent strain, WA8, or a virulent strain, JB14, two uninoculated wounds being made in each pod as controls. The experiment was performed in triplicate and the results were recorded after 3 and 6 days (Table 5 and Pl. 1, figs. 1, 2).

These results show that with a weakly virulent strain attack of pods has been promoted by low temperature. This supports the assumption that with the weakly virulent strain the browning of host tissue is limiting attack, since the rate of browning is reduced at lower temperatures. With the virulent strain this mechanism of host resistance is overcome at all the temperatures tested and the rate of attack is that which would be expected if temperature were having a direct effect on the growth rate of the fungus. However, it is possible that the effect of low temperature on other host processes may assist attack by the weakly virulent isolate.

Cultures of *P. palmivora* on carrot agar showed a progressive reduction of growth rate with decrease in temperature from 30° C. to 15–17° C.; thus the effect of low temperature in increasing the rate of rotting of pod tissue by a weakly pathogenic strain occurred in spite of a considerable reduction in growth rate.

Table 5. *Effect of temperature on growth of* Phytophthora palmivora *in cocoa-pod tissue* (*var.* ICS 60)

Strain	Temperature (° C.)	Average diameter of lesion after 3 days (cm.)	Average diameter of lesion after 6 days (cm.)
WA 8 (weakly virulent)	30	No lesion	No lesion
	25	No lesion	No lesion
	15	No lesion	1·5
JB 14 (virulent)	30	4·8	Lesions merged
	25	4·4	Lesions merged
	15	1·5	4·8



Text-fig. 1. Apparatus for maintaining cocoa pod at constant oxygen tension in closed space. The pods were suspended from the lids of 4½ l. jars (*G*), filled with the gas mixture and sealed except for a single outlet connected to a flask (*F*) containing oxygen, this flask in turn being connected to a reservoir of water (*R*). In the gas chamber the outlet had attached to it a length of polyethylene tubing with its end just dipping under water in a 25 ml. conical flask (*C*) to minimize diffusion of gases. The gas chamber also contained 100 ml. 30 % NaOH solution which absorbed the CO_2 given off in respiration. As oxygen was used up in respiration of the pod it was replaced by fresh oxygen from the flask *F*.

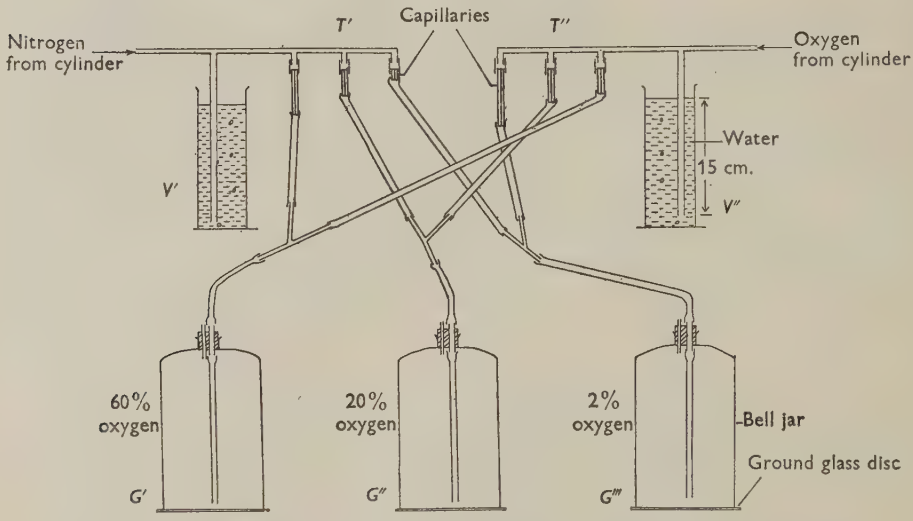
(*b*) *Effect of oxygen tension*

Inoculated pods were placed at different oxygen tensions in either a closed system (Text-fig. 1) or a continuous-flow system (Text-fig. 2).

The effects of oxygen tension on the growth of the virulent isolate JB 16 and the weakly virulent WA 8 on pods of the susceptible variety ICS 60 are shown in Tables 6–8 and Pl. 1, figs. 3–5. In these experiments, performed in Jamaica, a closed system was used and the required oxygen tensions were obtained by mixtures of oxygen and the

inert gas argon, since the more usual inert gas nitrogen was not available. The apparatus permitted only one pod per treatment in any one experiment but each experiment was repeated with similar results. In one experiment (Table 8) temperature was also varied. Pods in experiments recorded in Tables 6 and 8 were wound-inoculated and two additional uninoculated wounds were made on each pod; those recorded in Table 7 were inoculated in six places with a zoospore suspension placed in depressions made with the rounded end of a glass rod.

These experiments show clearly that low oxygen tension increases the rate of attack



Text-fig. 2. Apparatus for maintaining cocoa pods at constant oxygen tension in a continuous-flow system. A cylinder of oxygen and one of nitrogen were each connected to glass tubing T-pieces, the vertical arms of which dipped into the bottom of vessels (V', V'') containing columns of water 15 cm. high to maintain constant pressures; the other ends of the T-pieces were connected to three-pronged forks of glass tubing (T', T'') each 'prong' of which was connected to graduated lengths of capillary tubing, giving various flow rates by means of a bubble flow-meter in which the rate of ascent of a soap film was timed in a burette. Two capillary tubes, one on the nitrogen flow and one on the oxygen flow, were then connected by a Y-piece to produce a flow of mixed gas of the required composition, which was led into the bases of closed bell jars (G', G'', G''') containing the inoculated pods, through a second outlet at the top.

of the virulent isolate and permits attack by the normally avirulent one. Low temperature enabled the latter to attack at oxygen tensions above those permitting attack at higher temperatures.

In addition to the experiments carried out in sealed jars, where the accumulation of gaseous metabolic products might have influenced the results, four trials were made with a continuous-flow system, in which the inert gas used was nitrogen, at oxygen tensions of 60, 21 and 4% in one trial and 60, 21 and 2% in the other three: all were maintained at 25° C. In these experiments pods of the susceptible variety IMC67 were wound-inoculated with the weakly virulent isolate, WA8. The results of two

trials are shown in Table 9 and Pl. 1, figs. 6–8 (the remaining trials giving similar results to those of trial II in the table) and confirm those obtained with the closed system (Tables 6–8).

Growth rate of isolates on carrot agar was not significantly influenced by differences in oxygen tension, thus the effect of oxygen tension on ability to rot pod tissue cannot be attributed to a direct effect on growth.

Table 6. *Effect of oxygen tension* (*closed system, Text-fig.* 1) *on growth of a virulent isolate* (*JB* 14) *of* Phytophthora palmivora *on wound-inoculated pods of the susceptible var. ICS*60

Oxygen tension (%)	External appearance of lesions
10	Circular lesions, somewhat lighter brown in colour than those produced in air, approximately 5 cm. in diameter, darkening on exposure to air
5	Lesions similar to above, 5 cm. diameter
2·5	Lesions discernible only by slightly sunken edge and occasional light brown patches, browning uniformly on exposure to air, approximately 3 cm. in diameter

Table 7. *Effect of oxygen tension* (*closed system, Text-fig.* 1) *on growth of a virulent isolate* (*JB* 14) *of* Phytophthora palmivora *in pods of the susceptible var. ICS*60, *inoculated with zoospores*

Oxygen tension (%)	External appearance of lesions
60	Dark brown lesion. Av. diam. 2·1 cm.
21	Brown lesion. Av. diam. 3·3 cm.
4	Light brown lesion, darkening on exposure to air. Av. diam. 4·5 cm.

Table 8. *Effect of temperature and oxygen tension* (*closed system, Text-fig.* 1) *on growth of a weakly virulent isolate* (*WA* 8) *of* Phytophthora palmivora *in wound-inoculated pods of the susceptible var. ICS*60 *after* 7 *days*

Temperature (° C.)	Oxygen tension (%)				
	80	60	21	4	2
29–31	No lesion*	No lesion*	No lesion*	No lesion*	Spreading light brown lesion,† av. diam. 4·5 cm.
20–22	‡	No lesion*	Spreading brown lesion, av. diam. 3·9 cm.	Spreading light brown lesion,† av. diam. 4·2 cm.	‡
15–17	No lesion*	Spreading brown lesion, av. diam. 2·5 cm.	Spreading brown lesion, av. diam. 2·6 cm.	‡	Spreading light brown lesion,† av. diam. 2·5 cm.

* Narrow dark brown margin at point of inoculation.
† Lesion darkening on exposure to air.
‡ Treatment not set up.

Table 9. *Effect of oxygen tension (continuous-flow system, Text-fig. 2) on growth of a weakly virulent isolate (WA8) of* Phytophthora palmivora *in pod tissue of the susceptible var. IMC67*

Oxygen tension (%)	Trials I	Trials II
60	No lesion*	No lesion*
21	No lesion*	No lesion*
4	No lesion*	‡
2	‡	Light brown lesions, av. diam. 4·5 cm., darkening on exposure to air

* Narrow dark margin at point of inoculation.
‡ Treatment not set up in this trial.

Trial II was repeated twice with similar results.

DISCUSSION

The results of the present study support the pectolytic enzyme theory of parasitism (Brown, 1955).

P. palmivora on autoclaved potato blocks (p. 725) produces an active pectolytic enzyme which macerates potato tissue (p. 726) and, under particular conditions, cocoa-pod tissue (p. 727). The production of pectolytic enzymes by *P. palmivora* on autoclaved potato blocks, however, does not necessarily indicate a similar behaviour on living cocoa-pod shell tissue. Attempts to demonstrate pectolytic enzymes in rotted cocoa-pod tissue have not been successful (p. 725). This could be due to failure by the fungus to produce such enzymes under these conditions, but is more likely to result from destruction or inactivation of the enzymes by the host tissue. Thus the pectolytic enzymes secreted by the advancing hyphal tips could bring about local maceration of host tissues, but subsequent inactivation of the enzyme would prevent its accumulation in the tissue.

With brown rot of apples due to *Sclerotinia fructigena*, J. S. Cole (1956) was unable to demonstrate pectolytic enzymes in rotted apple tissue, which nevertheless showed a loss of pectin. Apple juice, if allowed to brown by the action of the naturally occurring oxidase, rapidly deactivated pectolytic enzymes prepared from *Botrytis cinerea* or *Sclerotinia fructigena* but deactivation did not occur if the oxidase system was inhibited by sodium cyanide. Thus the lack of demonstrable pectolytic enzyme is explained on the basis of the deactivation of excess enzyme by the brown products which develop during the rotting process. Byrde (1957, 1960) has suggested the polyphenol oxidase system as a resistance mechanism of apples to *S. fructigena*. He has shown that fast-browning varieties of apple are more resistant than slow-browning varieties. Among the polyphenols tested by Byrde, giving deactivation of pectolytic enzyme, was leucocyanidin prepared from cocoa tissue.

P. palmivora produces a firm brown rot of cocoa-pod tissue, similar to that produced in apple by *S. fructigena*. The brown products have been shown to be due to the oxidation of polyphenols by polyphenol oxidase (p. 728) as in the apple. Extracts of

cocoa-pod tissue browned during mechanical damage deactivate pectolytic enzymes of *P. palmivora* (p. 727), but if the process of browning is inhibited by sodium dithionite, this deactivation does not occur. Cocoa-pod tissue which has been allowed to brown after mechanical damage is more resistant to maceration by enzymes of *P. palmivora* (p. 727) than is similar tissue in which browning has been prevented by the addition of sodium dithionite. A comparison between a variety reported to show resistance to artificial inoculation and a known susceptible variety showed that pod tissue of the former both browned more rapidly and offered greater resistance to maceration (p. 727).

Thus the interplay of oxidizing and pectolytic enzyme systems discussed by Brown (1955) may well operate for *P. palmivora* on cocoa-pod tissue. Brown suggests the possibility that with some fungi on some hosts the prompt action of the oxidizing system brought into play by the initial attack may effectively prevent further attack by the parasite.

This idea has been further explored with an avirulent strain of *P. palmivora* on cocoa-pod shell tissue. On the hypothesis that success or failure of attack by the parasite depended on the balance between oxidizing and pectolytic systems, the effects of factors which it was thought would alter this balance in favour of the parasite were investigated. It was shown (p. 730) that both low temperature and low oxygen tension, factors known to reduce polyphenol oxidase activity, allowed rotting of cocoa pods by a normally avirulent strain of *P. palmivora*, which normally produces only a dark margin around the point of inoculation but shows no further attack.

Since other processes of both host and parasite will be affected by alteration of temperature and oxygen tension, the possibility that the disturbance of some other systems causes this promotion of attack cannot be excluded. Nevertheless, the disturbance of the balance between the pectolytic system of the parasite and the polyphenol oxidase system of the host offers the simplest explanation. Results of experiments on the effect of temperature and oxygen tension on growth of *P. palmivora* in culture make it unlikely that their direct effects on the parasite would promote attack.

Thus the results of this investigation are consistent with the view that in attack of cocoa pods by *P. palmivora* the parasite is able to penetrate by the production of pectolytic enzymes; that excess enzyme is deactivated by the products of the action of the polyphenol oxidase system of the host (this system coming into action only after some degree of disorganization of the host cell content) and that resistance to attack may be achieved by the host if the activity of its polyphenol oxidase system is great enough to deactivate the pectolytic enzyme of the parasite before penetration is achieved.

If further investigation confirms that the polyphenol oxidase system is a deciding factor in host resistance, then it seems likely that a sound basis may be provided for the selection of resistant material and for the breeding of resistant varieties.

I wish to express my thanks to Dr L. E. Hawker for helpful advice throughout these investigations and for assistance in the preparation of the manuscript. I am also indebted to Prof. E. W. Yemm, Botany Department, University of Bristol, and Prof. A. D. Skelding, Botany Department, University College of the West Indies, for placing the facilities of their departments at my disposal, and to Dr B. F. Folkes, Botany Department, University of Bristol, Dr R. J. W. Byrde, Long Ashton Research

Station, Bristol, and Dr M. Hudson, Botany Department, University College of the West Indies, for helpful advice. This work forms part of an investigation submitted for the Degree of Ph.D. of the University of Bristol and was made possible by a grant from the Government of Trinidad and Tobago.

REFERENCES

ASHOUR, W. A. (1954). Pectinase production by *Botrytis cinerea* and *Pythium de Baryanum*. *Trans. Brit. mycol. Soc.* **37**, 343.

BROWN, W. (1915). Studies in the physiology of parasitism. 1. The action of *Botrytis cinerea*. *Ann. Bot., Lond.*, **29**, 313.

BROWN, W. (1955). On the physiology of parasitism in plants. *Ann. appl. Biol.* **43**, 325.

BYRDE, R. J. W. (1957). The varietal resistance of fruits to brown rot. II. The nature of resistance in some varieties of cider apple. *J. hort. Sci.* **32**, 227.

BYRDE, R. J. W. (1960). The role of oxidised polyphenols in varietal resistance of apples to brown rot. In *Phenolics in plants in health and disease*, pp. 95–99. Oxford: Pergamon Press.

COLE, J. S. (1956). Studies in the physiology of parasitism. XX. The pathogenicity of *Botrytis cinerea*, *Sclerotinia fructigena* and *Sclerotinia laxa*, with special reference to the part played by pectolytic enzymes. *Ann. Bot., Lond.*, N.S., **20**, 15.

COLE, M. (1958). A comparative study of the mechanisms by which certain fungi and bacteria rot apple fruit. Ph.D. Thesis, London University.

DE BARY, A. (1885). Ueber einige Sclerotinien und Sclerotien-krankheiten. *Bot. Z.* **44**, 377.

DINGLE, J., REID, W. W. & SOLOMONS, G. L. (1953). The enzymic degradation of pectin and other polysaccharides. II. Application of the 'cup-plate' assay to the estimation of enzymes. *J. Sci. Fd Agric.* **4**, 149.

GRIFFITHS, L. A. (1958). Phenolic acids and flavonoids of *Theobroma cacao* L. Separation and identification by paper chromatography. *Biochem. J.* **70**, 120.

GUPTA, S. C. (1956). Studies in the physiology of parasitism. XXII. The production of pectolytic enzymes by *Pythium de baryanum* Hesse. *Ann. Bot., Lond.*, **20**, 179.

HOLLIDAY, P. (1954). Control of black pod and witches' broom in Trinidad. *Report of the Fifth Conference of the Inter-American Technical Cocoa Committee*, p. 10.

MENON, K. P. V. (1934). Studies in the physiology of parasitism. XIV. Comparison of enzymic extracts obtained from various parasitic fungi. *Ann. Bot., Lond.*, **48**, 187.

PADWICK, G. W. (1956). Losses caused by plant diseases in the colonies. *Phytopath. Pap. Commonw. Mycol. Inst.* no. 1.

RORER, J. B. (1910). The relation of black rot of cocoa pods to the canker of the cocoa trees. *Bull. Dep. Agric. Trinidad*, no. 9.

SPENCE, J. A. (1961). Black-pod disease of cocoa: I. A comparison of isolates of *Phytophthora palmivora* (Butl.) Butl. *Ann. appl. Biol.* **49**, 717.

WARD, H. M. (1888). On a lily disease. *Ann. Bot., Lond.*, **2**, 319.

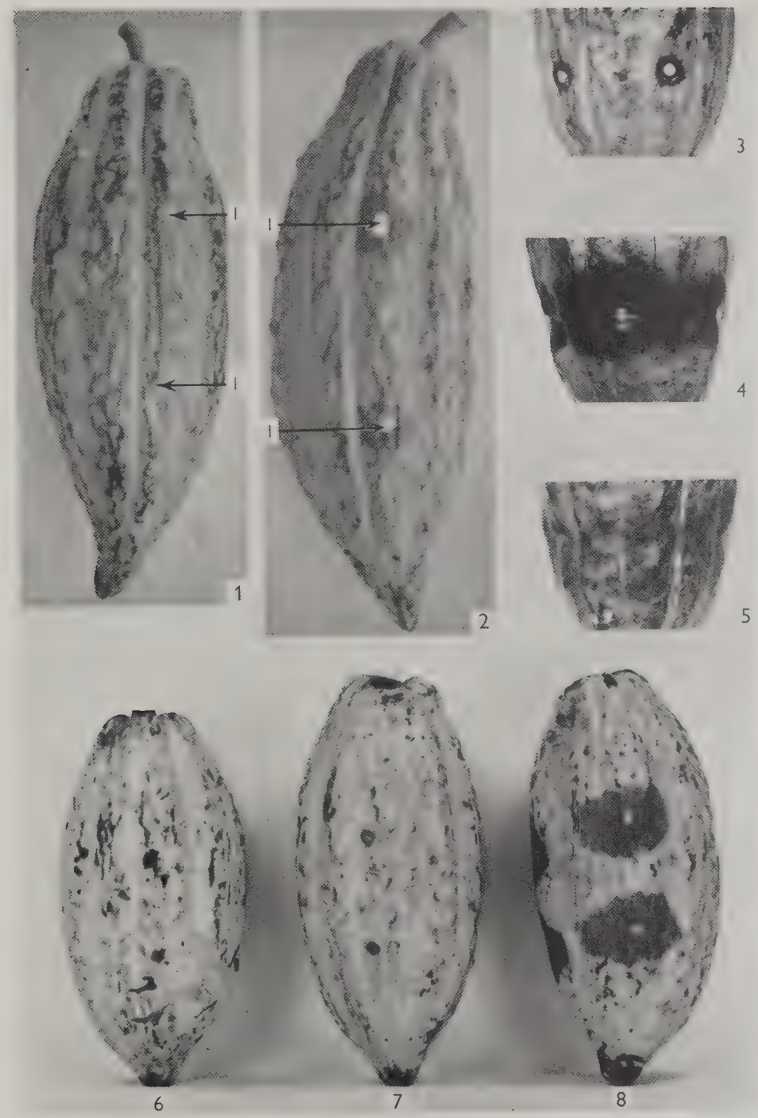
EXPLANATION OF PLATE

Cocoa pods wound-inoculated with isolates of *Phytophthora palmivora*

Figs. 1, 2. Pods of var. ICS 60 inoculated with isolate WA 8 of *P. palmivora*, incubated for 6 days at 30 and 15° C. respectively. I = site of inoculation. Note failure of fungus to invade tissues at 30° C. (fig. 1) and dark circular lesion surrounding inoculated wounds in pod incubated at 15° C. (fig. 2).

Figs. 3–5. Pods of var. ICS 60 inoculated with isolate WA 8, incubated at 20–22° C. for 7 days, at oxygen tensions of 60, 21 and 4% of that of the atmosphere respectively. Note restricted lesion at 60% tension, spreading lesions at 20 and 4% tensions.

Figs. 6–8. Pods of var. IMC 67 inoculated with isolate WA 8, incubated at 25° C. for 7 days, at oxygen tensions of 60, 21 and 2% of that of the atmosphere respectively. Note narrow dark margins produced round sites of inoculation at 60 and 21% tensions and spreading lesions (which darkened only after restoration of pods to normal atmosphere) surrounding sites of inoculation at 2% oxygen tension.



J. A. SPENCE

(*Facing p.* 734)

Ann. appl. Biol. (1961), **49**, 735–736
Printed in Great Britain

Reviews

The Mites of Stored Food. By A. M. Hughes. *Ministry of Agriculture, Fisheries and Food, Tech. Bull.* no. 9. Pp. vi + 287. London: H.M. Stationery Office. 1961. 17*s.* 6*d.*

Identifying mites is not among the easier tasks of the biologist. These animals are so small that the appropriate mounting of specimens and microscopy of high quality are often critically important. Certain species are difficult to distinguish from other closely related ones; some show a striking degree of variation; sometimes it is hard to decide which of these phenomena is concerned. These complexities are matched by those of the systematic literature, which is often confused, contradictory and hard to get.

Dr Hughes has had many years' experience in studying and identifying mites collected by the Infestation Control staff of the Ministry of Agriculture, Fisheries and Food. Fortunately for other biologists concerned with this fauna, she produced in 1948 a comprehensive illustrated monograph which enabled the determined non-specialist to put names to most of the species concerned. Since this earlier work has been out of print for some time, and in some respects out of date, and the closely guarded copies that are to be seen here and there are considerably worn, the revised edition, in hard covers, is very welcome indeed.

The present book deals with eighty-six species of mites in twenty families. As in the earlier edition, the layout follows the systematic order. Each group is defined, keys are provided where appropriate, each species is described rather fully, with illustrations, and brief notes are added on habits and world distribution. The 385 illustrations are line drawings of whole mites and of anatomical details at high magnification, excellently clear and accurate. After a brief introductory chapter on the systematics of the mites as a whole, about half the book is devoted to the sub-order Sarcoptiformes, particularly the section including the flour mite and other well-known store pests. Next, the Trombidiformes are represented by various predatory forms, notably species of *Cheyletus* which prey upon pests like the flour mite. Finally come the Parasitiformes, including chiefly the brown-coloured, fast-moving 'gamasid' mites, many of which prey upon other mites or insects or live parasitically upon vertebrates. The work concludes with six pages of bibliography, a short but valuable appendix on the preparation of specimens, and an index to species and other taxa.

Many changes in nomenclature have been incorporated in this revised edition. For example, the gamasid formerly called *Typhlodromus tineivorans* now appears as *Melichares tarsalis*, *Zercoseius gracei* becomes *Kleemania plumigera* (it should be *Kleemannia*, incidentally), *Tyrolichus* is added to *Tyrophagus*; the flour mite is now *Acarus siro*, *Tyroglyphus* having been declared an Invalid Name by the International Commission on Zoological Nomenclature. The author's way of dealing with some of the systematic technicalities may provoke a raising of taxonomic eyebrows. The applied biologist will not be too concerned about this, so long as it does not bring down another shower of revised names upon his head. His primary concern is to attribute his specimens to the right species; which of the alternative names he uses for any species is of secondary importance, though his desire for stability in this may conflict with a reluctance to be out of date. Here Dr Hughes, in up-to-date terms, provides the most generous assistance to guide him in his task of identification.

M. E. SOLOMON

New Approaches in Cell Biology. Ed. by P. M. B. Walker. Pp. viii + 208, illus. London and New York: Academic Press. 1960. £2. 2*s.*

In this book are published papers read at a symposium held during the Fifteenth International Congress of Zoology in 1958. The fourteen papers cover a wide range of topics, although the emphasis is almost entirely on the animal cell.

Perhaps the simplest way of describing the book is to list the topics: nuclear transfer of embryonic cells of the amphibia; cellular inheritance as studied by nuclear transfer in amoebae; lampbrush chromosomes; the morphology of developing systems at the ultramicroscopical level; the origin of the nucleus after mitotic cell division; labelled antibodies in the study of differentiation; a biochemical approach to cell morphology; paper chromatography in relation to genetics and taxonomy; the transfer mechanisms in active transport; the matching of drugs to tumours; the cytochemistry of proteins; the cytochemistry of nucleic acids; the interference microscope as a cell balance; flying-spot microscopy.

As might perhaps be expected in a book covering so many subjects by different authors, the quality is very uneven. Some of the papers read very easily and should present no difficulty to any intelligent biologist; others are rather laboured, and so technical that they seem to have been written primarily for specialists in their particular fields. This is a pity, for the book can hardly have been intended for the specialist interested in only one or a few of its papers. Its particular value lies in the way in which it brings to the notice of biologists recent important research in fields related to but different from their own, and all the papers should have been written with the general biologist in mind. I should point out that my difficulties bore no particular relationship to my familiarity with the subject. Some of the most interesting and easily readable papers were on topics almost new to me; while some of the hardest going was on fairly familiar ground.

Nevertheless, all the papers are well worth reading and many biologists, especially physiologists, embryologists and geneticists, will find much to interest them in this book, which will give them a picture of recent fields of advance in a very important sector of biology. The extensive reference lists which appear after some of the papers will be found particularly valuable, as will the extensive author index.

I have one strong criticism of production. The book is printed on rather stiff paper which refuses to lie flat, and I found it a very irritating book to handle while reading. J. L. CROSBY

Milk: The Mammary Gland and its Secretion. Two volumes. Edited by S. K. KON and A. T. COWIE. Pp. 515 and 423. New York and London: Academic Press. 1961. Volume I, 100*s*. Volume II, 86*s*.

The thirty contributors to these volumes are drawn from workers engaged in research in Europe and America, and the result is an authoritative and up-to-date presentation of knowledge covering the subject described in the title. The editors are well-known workers in the fields of Nutrition and Physiology, respectively, and in their foreword to the book acknowledge the association of Dr J. S. Folley, their colleague at the National Institute for Research in Dairying with the initial planning of the book.

Volume I deals with the subject in four parts:

(i) The Mammary Gland: Morphogenesis, Structure and Growth.
(ii) Functional Physiology of the Mammary Gland.
(iii) Lactation in Man and Farm Animals.
(iv) Biochemical Activities of the Mammary Gland.

Volume II is confined to the Nutrition of the Lactating Animal, and the Nutritional Value of Milk.

To those to whom milk means only an agricultural product it should be pointed out that the book covers a much wider field. For example, Volume II contains contributions on the Nutrition of the Lactating Woman; Dietary Requirements for Lactation in the Rat and other laboratory animals; and Human Milk and Cows' Milk in Infant Nutrition.

The book is intended for the research worker rather than the learner. Even so, many technologists concerned with the production of milk and its use in nutrition will find it a valuable work of reference.

It is very well produced and contains Author and Subject Indexes, together with exhaustive bibliographical references. G. T. MORGAN

Encyclopaedia of Plant Physiology. (*Handbuch der Pflanzenphysiologie*.) Edited by E. RUHLAND. Volume v (in 2 parts). The assimilation of carbon dioxide. Pp. 1013 and 868. Berlin: Springer-Verlag. 1960. Price DM. 530.

At a time when the literature on photosynthesis, and particularly that on biochemical and biophysical aspects of the process, is expanding rapidly, the average worker interested in the subject has increasingly to depend upon review articles to supplement and direct his reading of the original literature. This demand for partially predigested information has been adequately met by the various reviewing journals only in those areas of the subject where advance has been most rapid. There remain other large areas covered in a widely scattered literature in which advance has been steady but less spectacular and which are rarely subjected to review.

It would appear to be the aim of Volume v of the *Encyclopaedia of Plant Physiology*, as the title implies, to give a complete coverage to the whole of the literature pertinent to carbon dioxide assimilation as it existed at a point in 1958, extended in some articles by addenda to 1960. This also was substantially the object of *Photosynthesis and Related Phenomena*, by E. I. Rabinowitch, publication of which was completed in 1956. It may perhaps be questioned whether the time is yet ripe for a further expensive work covering similar ground. The approach used in the design of the two works, however, differs fundamentally. Rabinowitch was the sole author of his remarkable monograph written over a period of some seventeen years at a time of rapid development in the subject. The problem he had to overcome was to assimilate new findings into the framework of his text as it was in preparation. It is a tribute to the impartiality of the author in discussing opposing theories that this assimilation was in every case possible.

The volume under review is, by contrast, the product of the simultaneous work of 41 authors of 68 articles. All are well known in their own fields and most are active contributors to the current research literature.

The subject matter is divided into five sections: I. Introduction, II. Carbon dioxide and carbonic acid, III. Photosynthesis in green plants, IV. Photosynthetic bacteria, V. Chemosynthesis. The second section surveys, in six articles, the chemical and physical factors which determine the availability of carbon dioxide to plants in air and natural waters. Consideration of green plant photosynthesis follows logically and occupies some 70 % of the text of the volume. The literature on historical biochemical, photochemical, physiological and ecological aspects of the process is exhaustively reviewed in a long series of 55 articles. It would clearly be impossible to attempt even a cursory examination of the whole of this formidable section, and selection of individual articles for discussion would not do justice to their uniformly excellent standard. One consequence of the extensive subdivision used here is that overlapping and repetition of the subject matter of articles frequently occurs. B. Kok, for example, in a very able review on 'Efficiency of photosynthesis' discusses the culture of algae and the manometric methods used in the measurement of their photosynthetic rates and has occasion to refer to the work of J. Myers. He was apparently unaware that Myers has also contributed an article on these subjects in this volume. Similarly E. Steeman-Nielson in an article on 'Uptake of carbon dioxide by the plant' surveys essentially the same literature as M. G. Stalfelt in a subsequent article on 'Carbon dioxide' (as an external factor determining photosynthesis.) These two examples among many confirm the impression that cost has not been a major limiting factor in the production of this volume and authors have been allowed a loose editorial rein within their allotted subjects.

The penultimate section of the volume consists of one article by H. Gest and M. D. Kamen on 'Photosynthetic bacteria'. Although it is surprising that the relative amount of space devoted to this important group of organisms is so small the article provides a concise summary of their metabolic processes with an addendum to the bibliography extending the survey to 1960. These photo-autotrophs are further considered in the final section on 'chemosynthesis' where their relations to the chemo-autotrophs is discussed from the viewpoint of comparative biochemistry.

The usefulness of an encyclopaedia is determined to a great extent by the completeness of its index. The first impression here was that a subject index of 38 pages, separate from the author index, was disproportionately short for a volume of this length. In practice, although a number of omissions were detected, the cross-indexing proved to be sufficiently detailed for all normal usage. This index is duplicated in English–German as well as German–English versions.

The price of this volume must undoubtedly place it beyond the reach of most scientific

workers and the smaller departmental libraries. Others may be deterred from purchase by the fear that it will rapidly become obsolete as new findings emerge. This consideration should not be allowed to obscure the value of the volume as a work of reference to an extensive range of permanent factual information. Moreover, in the more rapidly expanding regions of research in photosynthesis it will provide a background against which the newer findings can be orientated and evaluated.

H. E. DAVENPORT

Soil Biology. By WILHELM KÜHNELT. (Translated from the German by Norman Walker.) Pp. 397, 77 figures, 4 plates. London: Faber and Faber Ltd. 1961. 45*s*.

For the last ten years soil zoologists have been familiar with Prof. Wilhelm Kühnelt's *Bodenbiologie*, and Dr Norman Walker's English translation will therefore be widely welcomed. This edition is, however, not simply a translation of the German text published in 1950; it has been brought up to date, and contains much material published between 1950 and 1959.

This is not a 'popular' book, though it should not offer much difficulty to any reader with some biological training. It should be useful to university students of zoology and ecology as well as being of interest to pedologists and others working with the soil.

The first chapter gives a critical review of the different techniques for collecting soil animals, and for sampling, quantitatively and qualitatively, populations in various habitats. Almost every research worker in this field has produced his own technique; references to detailed descriptions are given.

The survey of the soil animals (130 pages) includes a general account of the fauna. No attempt is made to include information which will allow the reader to identify his collections except into the large groups. It would perhaps have been useful to have included more references to more actual works to assist in identifications, rather than to refer only to the 'normal animal identification books' with which some readers may be unfamiliar. Some of the 77 line drawings are rather undistinguished, though most are adequate. There are 4 plates. The account of the biology of the animals and of the relationship with their environment is excellent, and forms a major contribution to ecological knowledge.

The bibliography occupies 55 pages and contains over 1200 titles. There is an index of names of animal species, but no subject index.

Prof. Kühnelt's book shows how much work, particularly on the continent of Europe, has been done in recent years. He also takes pains to stress that we still have much to learn on most fundamental problems. It is to be hoped that this English edition will help to encourage more biologists to work on this important and difficult subject.

K. MELLANBY

Insect Life and Insect Natural History. By S. W. FROST. Pp. vi + 526. Dover Publications Inc. New York. 1959. $2.25.

Prof. Frost's earlier book, *General Entomology*, published in 1942, was an admirable attempt to provide an entomological textbook which would be valuable to that mass of students who wish to know something of insects without becoming entomologists. It is a pity that the present volume, which appears to be a new book, is in fact only a very slightly revised paper-back re-issue of the original publication.

The bibliographies following each chapter have been amplified by only one or two references, so the mass of literature of the past decade has had no impact on the original text. The inclusion of an Appendix containing keys to some groups of larvae, a summary of groups of subterranean insects and a list of lepidopterous leaf-miners seems quite out of place and keeping with the purpose of the book.

N. W. HUSSEY

HEREDITY

AN INTERNATIONAL JOURNAL OF GENETICS

VOLUME 16 NOVEMBER 1961 PART 4

Annual subscription £4 (U.S.A. & CANADA $14.00)
Single part 25*s.* (U.S.A. & CANADA $4.00)

OLIVER AND BOYD LTD.
Tweeddale Court, 14 High Street, Edinburgh, 1

THE ANNALS OF APPLIED BIOLOGY

The Annals of Applied Biology is conducted by the Association of Applied Biologists and is published by the Cambridge University Press. The Association exists to further the study of all aspects of Biology and to correlate pure science with practice. Meetings are held about six times a year for informal discussion and the reading of papers.

The annual subscription to the Association, which includes a copy of the *Annals*, is £2, becomes due on 1 Jan. of each year and should be paid to the Treasurer.

The price of the *Annals* to non-members of the Association is £5 per volume or $17.50 in U.S.A. Such subscriptions (payable in advance) and all communications respecting the publication should be sent to the Cambridge University Press, 200 Euston Road, London, N.W. 1, or in U.S.A. to the American Branch of the Press at 32 East 57th Street, New York, 22. Single numbers 30s. or $5.00.

Claims for missing numbers should be made within the month following that of regular publication.

Quotations for available back volumes may be obtained on application to the Cambridge University Press.

Notice to Contributors

Manuscripts for Publication

Contributors should address all material to Mr R. W. Marsh, Research Station, Long Ashton, Bristol. Manuscripts submitted for publication must be typewritten, double-spaced with ample margins, on one side only of white opaque paper approximately 13 × 8 in. It is understood that they are not offered to any other Journal for prior or simultaneous publication. Preference will be given to Members' papers: papers from non-members must be communicated by a Member for consideration by the Editors.

Owing to the demand for space and the high cost of printing, authors must drastically condense their manuscripts, in the general interest of all contributors. Numerical results should be presented either as tables or as diagrams, but not both; only such tables and illustrations as are essential can be published. Papers must start with a summary and conform to the usages of the *Annals* in all typographical matters. Contributors will be responsible for any excess over the usual charges allowed for corrections. Papers over 8000 words can be published only in exceptional circumstances. Quantitative biological data too extensive for complete publication should be deposited with the Librarian, British Museum (Natural History), Cromwell Road, London, S.W. 7.

References

References must be listed at the end of the article according to the 'Harvard System' as follows: name and initial(s) of author; year of publication in brackets, further distinguished by the addition of small letters *a*, *b*, *c* to the date where more than one paper published by the same author(s) in the same year is cited; exact title of paper; contracted title of periodical as given in *World List of Scientific Periodicals*; volume number in arabic figures; beginning page number of article. In the text references should be denoted by giving the name of the author(s) with the date of publication in brackets, e.g. (Brown, 1937), (Brown, 1937*a*; Jones & Smith, 1942*a*, *b*). In the References *all* names should be given in full but, where more than *two* collaborating authors are quoted in the text, the names are printed in full only at the first citation; after that the first name is followed by *et al.*

Illustrations

Text illustrations accompanying the papers should be drawn on smooth white Bristol board in Indian ink about twice the size of the finished block. Shading must be indicated by lines or dots. Graphs should be drawn boldly in Indian ink on white Bristol board or on white graph paper with pale-blue lines. All lettering of illustrations or graphs should be inserted clearly in pencil.

Separates

Twenty-five separates of each paper are provided free.

Printed in Great Britain at the University Press, Cambridge (Brooke Crutchley, University Printer)